2008

Understanding cold acclimation in Medicago truncatula

Babita Thapa
Iowa State University

Understanding cold acclimation in *Medicago truncatula*

by

Babita Thapa

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Crop Production and Physiology

Program of Study Committee:
Allen D. Knapp, Co-major Professor
E. Charles Brummer, Co-major Professor
Rajeev Arora
Kenneth J. Moore
Jeremy Singer

Iowa State University
Ames, Iowa
2008

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 1. GENERAL INTRODUCTION

Introduction

M. truncatula is a close relative to alfalfa, the fourth most widely grown agricultural crop in the United States (Zhang et al., 2005). Nearly half of the approximately 9.04 million hectares (http://usda.gov/nass/) of alfalfa is concentrated in the upper Midwest and the northern Great Plains (Riday and Brummer, 2002). Winter hardiness is the most important trait for alfalfa grown in northern climates because it influences stand longevity, quality and production (Ipson 1991) and has been predicted by measuring fall dormancy. Fall dormancy scores of cultivars are determined by measuring vertical growth following the last fall cutting, with a score 1 and 11 representing the most fall dormant and non-dormant, respectively (Teuber et al., 1998). Although fall dormant cultivars are desirable for survival in subsequent years, they generally produce little yield in autumn. In contrast, non-dormant cultivars produce higher yield in the establishment year but are prone to winter kill, which subsequently reduce yield. Therefore, improvement of both, fall dormancy and winter survival are important to improving yield of alfalfa in the northern region.

In general, phenotypic correlation between fall dormancy and winter survival are strongly positive in alfalfa cultivars with varying fall dormancy ratings (Schwab et al., 1996). In contrast, a weak (Brummer et al., 2000) or no association (Busbice and Wilsie, 1965) between the two traits was observed in studies using F1 or F2 progenies indicating that fall dormancy and winter survival can be improved independently. Although there is considerable knowledge on morphological, developmental and molecular bases of winter

survival, and the acquisition of freezing tolerance in alfalfa (Castonguay et al., 2006), our basic knowledge in fall dormancy/cold acclimation is inadequate. Time of start and end of acclimation response and amount of cold acclimation contributing to survival are still unknown in alfalfa which impedes the progresses on manipulation of cold acclimation processes for autumn yield improvement (Brummer et al., 2000).

Physiological and genetic studies for important traits are complicated by autotetraploidy, a large genome size and allogamy in alfalfa (Thouquet et al., 2002). *M. truncatula* is a model species for studying legume biology because it has a small genome size, diploidy, autogamous fertilization, rapid reproductive cycle, and several available cultivars (Thouquet et al., 2002). There is also high degree of synteny between *M. truncatula* and *M. sativa* (Choi et al., 2004). With a total of approximately 190,000 expressed sequence tags (ESTs) available for use (May, 2004) and relative ease of discovering genes and their functions compared to alfalfa, *M. truncatula* could be used as a model for understanding physiological and genetic bases for differences in cold-acclimation in alfalfa.

Medicago truncatula (Barrel medic) is native to the region surrounding the Mediterranean Sea and an important forage crop in Australian and South African pasture (O'Neil and Bauchan, 2003). It is one of several annual *Medicago* species that are cultivated as a smother crop for weed suppression, green manure crop, cover crop in row crop production or short-season forage crop (O'Neill and Bauchan, 2000). Medic forage is higher in crude protein and lower in fiber than alfalfa (Sheaffer, 1998). In the north-central USA, annual medics can produce high yields of quality forage when grown as short season annual crops for autumn harvest (Zhu et al., 1998). Although *M. truncatula*

does not survive winter conditions in the northern USA, it often encounters and survives frost, and therefore, may offer some clues about cold tolerance in alfalfa. *M. truncatula* is a model crop for studying the genetics legumes and has a potential as a short-season annual crop to supply forage when traditional supplies are inadequate.

Thesis Organization

This thesis is organized into five chapters. Chapter one is the general introduction. Chapter two is an article to be submitted to *Crop Science* entitled "Growth variation among *Medicago truncatula* accessions under simulated autumn conditions and their comparison with alfalfa". Chapter three is an article published in the *Journal of American Society of Horticultural Sciences* entitled "Applying freezing test to quantify cold acclimation in *Medicago truncatula* Gaertn." Chapter four is an article to be submitted to *Crop Science* entitled "Cold acclimation alters soluble sugar concentration and composition in *Medicago truncatula* Gaertn." Chapter five is the general conclusion of this project.

Chapter two compares the variation in root and shoot dry weights among *M. truncatula* accessions between simulated autumn and constant growth conditions and compares the results with alfalfa fall dormancy check cultivars. Chapter three describes a laboratory screening protocol to quantify cold acclimation in *M. truncatula* by applying a freezing test and explains differential cold acclimation regimes applied in the study. Chapter four describes the differences in soluble sugar concentration and composition at different acclimation regimes and their association with freezing tolerance.

Literature review

Taxonomy

Medicago truncatula Gaertner belongs to the leguminosae family and is commonly known as Barrel medic. It is one of the annual legumes that are cited as "medics" in many literatures. There are three subspecies of *M. truncatula* divided based on pod characteristics; ssp. *truncatula*, ssp. *tricycla* and ssp *longeaculata* (Lesins and Lesins, 1979).

Habitat and distribution

Lesins and Lesins (1979) described *M. truncatula* as an omi-mediterranean species native to Northern Africa (Tunisia, Algeria, Morocco), Asia-Temperate (Cyprus, Jordan, Turkey, Georgia, Armenia) and Europe (Ukraine, Greece, Italy, France, Spain). *M. truncatula* is an important forage crop in Australia, grown in southern Australian semi-arid agricultural zones where the climatic conditions match those of the Mediterranean region; annual rainfall between 250-500 mm, mild winters with occasional frosts, and summer temperatures remaining above 40°C in inland areas. The introduction of the species in Australia is thought be as an accidental contaminant with other seeds and with domestication of animals although it was naturalized following European migrations in the 18th century to the rest of the world (Crawford et al., 1989).

M. truncatula is adapted to a wide soil texture types, sandy to clayey and pH, 6 to 8. It is intolerant to lower soil pH and low moisture because plants are not deep rooted (Evans et al., 1990).

M. truncatula contains diverse ecotypes. Spatial analysis of a population reveals that subdivisions can be found in populations between transects separated by 10-50 meters (Bonin et al., 1996) and within a sub population spatial autocorrelation can be found among individuals located up to 7 meters apart (Bonin et al., 2001). In a recent study, Ellwood et al. (2006) observed substantial diversity in core collections of South Australian Research and Development Institute (SARDI) with identification of more than 90% of 192 randomly selected accessions as discrete genotypes, using six single sequence repeat (SSR) loci. Their data suggested the presence of unusually high dispersal of seeds in *M. truncatula* throughout the mediterranean region which could possibly be due to the animal or trade-related movements.

Wild accessions/ populations

There are a total of 5703 collections of *M. truncatula,* collected from the centers of origin and centers of diversity through the effort of four major organizations; AMGRC (Australian Medicago Genetic resource Centre), INRA (France, Institut National de la Recherche Agronomique), ARS-USDA (Agricultural Research Service, United States Department of Agriculture) and CBBC-LILM (Laboratoire Interactions legumineuses Microorganismes, Centre Biotechnologie, Technopole de Borj, Tunisia) (Greene et al., 2007). However, diversity of the collection does not equally represent the centers of origin. Almost 46 % of the total collected populations are from Morocco, Libya and Tunisia whereas the centers of origin, such as, north east mediterranean basin, south of Caucasus are under-represented. Thirty-four percent of wild populations come from 9 Eastern Mediterranean Basins and 20% of the *M. truncatula* collections came from six

countries of Southern Europe. The collection of *Medicago truncatula* at the South Australian Research and Development Institute (SARDI) is known as the world's oldest and the largest of all the centers (Ellwood et al., 2006).

Levels of genetic variation within *M. truncatula* populations may vary from little diversity when a population is composed of all homozygous genotypes, to a higher level when two or more heterozygous genotypes dominate the population (Bataillon and Ronfort, 2007).

Morphology, architecture and development

Moreau et al. (2006) has proposed a standard system for characterization of the vegetative stage for phenotypic analysis. Plant consists of: (1) main axis that can be either elongated or be organized in a rosette; and (2) branches of different orders (primary, secondary branches). Plant architecture is largely affected by cultural conditions; rosette (prostrate plant) when they are grown in low plant density and elongated (erect plant) with a high plant density or with low radiation. *M. truncatula* has epigeal emergence. After cotyledons are released, leaves are developed at axils of the main axis. The two successive leaves are alternatively placed, on one side and on the other side of the axis. Primary branches develop from the axil of the main axis leaves. The first primary branch appears at the axil of the first developed main axis leaf or the unifoliolate leaf. Secondary and tertiary branches develop at the axil of leaves of secondary and tertiary branches, respectively.

Floral ontogeny of *M. truncatula* is unidirectional in all whorls starting from the abaxial position of the flower with a high degree of overlap; just the opposite to

Arabidopsis where sequential development takes place (Benlloch et al., 2003). The authors divided floral meristem development into eight different stages and described each stage with the aid of colored scanning electron micrograph (SEM) photographs. Bucciarelli et al. (2006) developed a standardized numerical coding system that enables easy identification of developmental stages in *M. truncatula* to define growth. Their system was successful in distinguishing phenotypic changes resulting from growth under nutrient stress conditions. The chronological appearances of root, shoot, and flower development are detailed in the paper.

Seed are set in pods that are compact and spiky and contain numbers of seeds ranging from 5-8 on average depending on growth conditions. Seeds are covered by a hard-coat that needs to be scarified for imbibition and germination and can be stored for a long time (40 years) without losing their viability if seeds are kept intact in pods without threshing (Crawford et al., 1989). Depending upon the genotype and environmental conditions, seed hardiness may vary from 10 to 100% within a seed lot (Taylor, 1996).

Variation for quantitative traits

Genetic diversity in flowering time has been the most studied trait in *M. truncatula*. Crawford (1989) indicated that the range of flowering time was from 62 to 148 days for accessions originated from the Mediterranean basin planted in South Australian environment. A large variation in flowering time, branch length, and branch diameter and branch elongation rate was observed among 29 populations and lines (Julier et al., 2006). Although QTLs were detected for each trait in their study, strong ones (LOD= 10.2-18.9; R^2 = 28-59.2) were found for flowering time on chromosome 7 in their

multi-location trials. Bonnin, et al. (1996) compared molecular marker (RAPD markers) with 24 quantitative traits that are associated with seedling, vegetative growth and reproductive traits in two populations, in order to determine the relative importance of natural selection in the process of differentiation. They found that populations were diverse in terms of the quantitative traits that were mainly related to the traits of fitness (flowering time, pod production) than the markers. These results suggested that trait variation may relate to development of local adaptation or ecotypic differentiation in *M. truncatula* collections (Batallion and Ronfort, 2006). In a study, Walsh et al. (2001) evaluated growth and development periods of *M. truncatula* cultivars developed in southern Australia under a southeastern Wyoming climate. Cultivars flowered from 71 to 148 days from emergence in Australia while they flowered much earlier, within 36-73 days in Wyoming shortening the duration of growth and development.

Temperature, photoperiod and vernalization effects on flowering

Aitken (1955b) observed acceleration in flower initiation in Barrel medic by counting the nodal position of the flower cluster, using a commercial cultivar from Australia. She found that low temperature exposure at the seedling stage and a long photoperiod lowered the nodal position of the flowers, initiating earliness in flowering. Clarkson and Russell (1975) observed that 3 weeks of vernalization at 1°C gave the maximum effect on time of flowering in six annual medics, including *M. truncatula*. However, when vernalization exceeded 7 weeks, *M. truncatula* cv. Jemalong showed a very strong reversal response under a 12-hr photoperiod. High temperature also accelerated flowering time in Jemalong but only after the vernalization requirements

were met. The cultivar flowered in 38 days with vernalization and in 100 days without vernalization when gown at 21°C. Thus, flowering in *M. truncatula* appears to be controlled by interaction of three environmental factors: temperature, vernalization and photoperiod.

Abiotic stresses

(1) Water stress

M. truncatula is more drought-tolerant than soybean and peas but shows similar response to the stress as does alfalfa, both physiologically and biochemically (Rubio et al., 2002). Seedlings of *M. truncatula* grown under water stress conditions in a greenhouse showed a drastic reduction in the number of internodes (17%), number of primary branches (29%), shoot fresh weight (56%) and dry weight (48%) compared to non-stressed plants (Chebouti and Abdelguerfi, 2004).

(2) Salt stress

Veatch et al. (2004) studied variation for salt tolerance among greenhouse–grown *M. truncatula* accessions exposed to saline and nonsaline irrigation at the seedling stage. The authors compared the shoot biomass among the accession at different salinity levels with nonsaline conditions. The accessions that produced the highest fresh shoot biomass in saline conditions also produced highest in nonsaline conditions. The high correlation between the fresh biomass measured under two conditions indicated that there is no distinct physiological adaptation to salinity among the evaluated accessions.

(3) Cold stress

In the Mediterranean region, most vegetative growth occurs during winter. There are numerous studies focused on understanding cold response and physiological mechanisms for cold tolerance.

Hekneby et al. (2001) subjected 21-d old plants to 20/15 or 10/5°C day/night temperatures until 40 days to compare differences in growth parameters, proline, starch and sugar content among legumes. Root:shoot ratio increased in cold treated *M. truncatula* but the total dry matter, leaf area, specific leaf area, leaf area ratio did not differ between the two temperature treatments suggesting it to be the most cold tolerant among the annual legumes. Sanchez-Diaz et al. (2000) studied seedling survival in *M. truncatula* and other annual and perennial legumes grown at 10/5°C during 20 and 40 days periods. On average, almost 75% of the *M. truncatula* seedlings survived the cold treatments without a significant difference between growth durations. *M. truncatula* had the lowest relative water content among all the species evaluated at 40 days under treatment. The authors found that the annual legumes concentrated more total soluble sugars in stems than roots and leaves which was positively correlated to relative growth rate and suggested that the higher total soluble sugars may relate to higher cold tolerance. Antolin et al. (2005) studied growth, net photosynthesis rate and chlorophyll fluorescence induction kinetics among annual medics under cold acclimation (10°C) and nonacclimation regimes. They found that *M. truncatula* cultivar Paraggio developed at 10°C produced photosynthetic cold acclimation that was associated with a two-fold increase of quantum yield of photosystem 2 electron transport and with the activity of

stromal fructose-1, 6-bis-phosphatase (sFBPase). Growth at the low temperature produced higher dry matter and resulted in a 2-fold increase in stem and root dry matter without any changes in leaf dry matter than at 20°C.

Frost resistance in the *M. truncatula* cultivar Paraggio was assessed by growing it at 10/5°C and measuring the regrowth capacity after transferring to 20/15°C for 4 weeks. The authors concluded that the cultivar had an inefficient cold acclimation process because of impaired SPS activity and low starch reserves observed which resulted in poor regrowth in the cultivar. However, the ice nucleation temperature decreased in cold acclimated leaves compared to control (Hekneby et al., 2006). Brandsæter et al. (2000) investigated the effects of freezing temperatures on winter annuals to evaluate their survival and biomass potential. The *M. truncatula* cultivar Parabinga cold hardened at 2°C for 2 weeks had 100% plants survival at 0°C, 97% at -2°C while none of the plants survived at -9°C after they were at 18°C for 3 weeks in greenhouse. The authors suggested that the poor survival in the plants could be because they had already reached the flowering stage when frozen in the growth chamber.

Importance/ agricultural value

(1) Model species

Because most agriculturally important legume crops such as alfalfa, clover, pea and soybean are difficult to analyze genetically due to characteristics such as polyploidy (soybean, alfalfa), large genomes (pea, soybean) and lack of efficient methods for tansgenesis (pea, soybean), studying a model legume offers opportunities to analyze plant physiological processes, symbiotic and plant-pathogen interactions for the process of

atmospheric dinitrogen fixation (Penmetsa and Cook, 2000). *M. truncatula* demonstrates higher levels of nucleotide sequence conservation and similar genetic organization to alfalfa, pea, chickpea and clover which makes transfer of its genome information possible to these species (Doyle et al., 1996; Thoquet et al., 2002). Penmetsa and Cook (2000) reported efficient mutagenesis in *M. truncatula* with the development and characterization of three developmental mutants that could be useful tools for efficient molecular genetic studies in the model legume and for identifying genes that are associated with diverse developmental processes.

When primer pairs designed from expressed sequences tags (ESTs) from *M. truncatula* containing simple sequence repeats (SSR) were screened in alfalfa, high levels of polymorphism were detected for the markers indicating that the markers developed from *M. truncatula* could be valuable genetic markers for alfalfa (Eujay et al., 2004). Some attempts of isolating genes of desired alfalfa traits are already underway from *M. truncatula* using the molecular genetic linkage map. Sledge et al. (2005) studied 301 *M. truncatula* accessions from the USDA National Plant Germplasm System, in a hydroponics approach with a long-term goal of identifying aluminum (Al) tolerance genes to be used for alfalfa improvement. They found variation in relative root growth from 0.35 to 1.09 indicating that sufficient variation exists within the collection to select sensitive and tolerant accessions that could be used to identify QTL for Al tolerance.

(2) Animal food/ forage crop

M. truncatula is a potential pasture crop that produces high levels of good quality forage. In Southeastern Wyoming, *M. truncatula* cultivars; Caliph, Mogul and Paraggio

produced 1.6, 3.1 and 4.0 t ha^{-1} mean dry matter yield (3 years) when grown in the summer of southeastern Wyoming. The crude protein levels ranged from 197 to 198 g kg^{-1} and the relative feed values from 192 to 219 were comparable among the cultivars and other annual legumes. Based on these results on yield and forage quality, the authors suggest that most of the annual medic cultivars would be well suited for livestock production (Walsh et al., 2001).

(3) Cover crop

In organic farming, growing legume cover crops as living mulches are a sustainable weed-control strategy. Annual medics have a potential as a cover plants in corn production (De Haan et al. 1997, Jeranyama et al., 1998). Fisk et al. (2000) studied *M. truncatula* in a winter wheat/corn rotation to evaluate it for weed suppression ability in a no-till cropping system. The no-till seeded cover crops reduced density of winter annual weeds by 41 to 78% when compared with no cover control while the weeds dry weights were lowered by 80%. Dry weights of annual weeds before corn planting were reduced by 70% following the cover crop. With these results, the authors concluded that the *M. truncatula* has an excellent potential to reduce weed density and growth in no-till corn grain system.

The *M. truncatula* cultivar Salernes suppressed monocotyledon, dicotyledon and spring-germinated and annual weeds by the time of wheat (*Triticum aestivum* L.) anthesis but was less effective compared to other legumes due to lower survival in winter. De Haan et al. (2002) evaluated different annual medics including *M. truncatula* for upper Midwest agroecosytems to study desirable traits such as shade tolerance, growth habit

and potato leafhopper yellowing. None of the medics possessed of all the traits but all were present within the germplasms suggesting that development of cultivars for the region could be achieved through breeding.

In the Northern Great Plains, annual medics have potential as self regenerative pasture crops in rotation with wheat in integrated crop-livestock systems. In a study conducted by Walsh et al. (2001), annual medics including 3 cultivars of *M. truncatula* produced high forage quality and yielded high dry matter suitable for livestock production. Cultivars Caliph, Mogul and Paraggio produced 10491, 79227, and 9993 seed m^{-2}, respectively, averaged over three seasons in Wyoming conditions which were more than the minimum level (4000 seed m^{-2}) for a viable annual medic seedbank. The authors suggested that the levels of seed production of the evaluated cultivars are adequate for establishment of a viable seedbank that would allow regeneration for a number of years.

Literature Cited

Aitken, Y. 1955. Flower initiation in pasture legumes. III. Flower initiation in *Medicago tribuloides* Desr. and other annual medics. Australian Journal of Agricultural Research 6:258-264.

Antolín, M.C., M. Hekneby, and M. Sánchez-Díaz. 2005. Contrasting responses of photosynthesis at low temperatures in different annual legume species. Photosynthetica. 43:65-74.

Bataillon, T., and J. Ronfort. 2006. Evolutionary and Ecological Genetics of Medicago truncatula. The Medicago Handbook. (Available at: http://www.noble.org/MedicagoHandbook/pdf/EvolutionaryEcologicalGenetics.pdf).

Bonnin, I., J.M. Prosperi, and I. Olivieri. 1996. Genetic markers and quantitative genetic variation in *Medicago truncatula* (Leguminosae): A Comparative Analysis of Population Structure. Genetics 143:1795-1805.

Bonnin, I., J. Ronfort, F. Wozniak, and I. Olivieri. 2001. Spatial effects and rare outcrossing events in Medicago truncatula (Fabaceae). Molecular Ecology 10:1371-1383.

Brandsater, L.O., T. Smeby, A.M. Tronsmo, and J. Netland. 2000. Winter annual legumes for use as cover crops in row crops in Northern regions: II. Frost resistance study. Crop Sci. 40:175-181.

Brummer, E.C. 2004. Applying genomics to alfalfa breeding programs. Crop Sci. 44:1904-1907.

Brummer, E.C., M.M. Shah, and D. Luth. 2000. Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. Crop Sci. 40:971-977.

Busbice, T.H., and C.P. Wilsie. 1965. Fall growth, winterhardiness, recovery after cutting and wilt resistance in F2 progenies of Vernal X DuPuits alfalfa crosses. Crop Sci. 5:429-432.

Castonguay, Y., S. Laberge, E.C. Brummer, and J. Volenec. 2006. Alfalfa winter hardiness: A research retrospective and integrated perspective. Adv. Agron. 90:203-265.

Clarkson, N.M., and J.S. Russell. 1975. Flowering responses to vernalization and photoperiod in annual medics (*Medicago* spp.). Aus. J. Agric. Res. 26:831-838.

Crawford, J.E., A.W.H. Lake, and K.G. Boyce. 1989. Breeding annual *Medicago* species for semiarid conditions in southern Australia. Adv. Agron. 42:399-437.

Castonguay, Y., and P. Nadeau. 1998. Enzymatic control of soluble carbohydrate accumulation in cold-acclimated crowns of alfalfa. Crop Sci. 38: 1183-1189.

Chebouti, A.C., and A. Abdelguerfi. 2004. Effect of water stress on growth and forage yield of three species of medics. Cahiers Options Mediterraneennes 62:319-321.

Choi, H.-K., D. Kim, T. Uhm, E. Limpens, H. Lim, J.-H. Mun, P. Kalo, R.V. Penmetsa, A. Seres, O. Kulikova, B.A. Roe, T. Bisseling, G.B. Kiss, and D.R. Cook. 2004. A sequence-based genetic map of *Medicago truncatula* and comparison of marker colinearity with *M. sativa*. Genetics. 166:1463-1502.

Cunningham, S.M., J.A. Gana, J.J. Volenec, and L.R. Teuber. 2001. Winter hardiness, root physiology, and gene expression in successive fall dormancy selections from 'Mesilla' and 'CUF 101' alfalfa. Crop Sci. 41:1091-1098.

De Haan, R., C. Sheaffer, D. Samac, J. Moynihan, and D. Barnes. 2002. Evaluation of annual *Medicago* for upper midwest agroecosystems. Journal of Agronomy and Crop Sci. 188:417-425.

Ellwood, S.R., N. K. D'Souza, L.G. Kamphuis, T.I. Burgess, R.M. Nair, and R.P. Oliver. 2006. SSR analysis of the Medicago truncatula SARDI core collection reveals substantial diversity and unusual genotype dispersal throughout the mediterranean basin. TAG Theoretical and Applied Genetics 112:977-983.

Eujayl, I., M. Sledge, L. Wang, G. May, K. Chekhovskiy, J. Zwonitzer, and M. Mian. 2004. *Medicago truncatula* EST-SSRs reveal cross-species genetic markers for *Medicago* species TAG. Theoretical and Applied Genetics 108:414-422.

Gallardo, K., C. Le Signor, J. Vandekerckhove, R.D. Thompson, and J. Burstin. 2003. Proteomics of *Medicago truncatula* seed development establishes the time frame of diverse metabolic processes related to reserve accumulation. Plant Physiol. 133:664-682.

Garnier, E. 1992. Growth analysis of congeneric annual and perennial grass species. J. Ecol. 80:665-675.

Guy, C.L.and D. Haskell. 1987. Induction of freezing tolerance in spinach is associated with the synthesis of cold acclimation induced proteins Plant Physiol. 84: 872-878.

Fisk, J., O. Hesterman, A. Shrestha, J. Kells, R. Harwood, J. Squire, and C. Sheaffer. 2001. Weed suppression by annual legume cover crops in No-Tillage Corn. Agronomy Journal 93:319-325.

Haagenson, D.M., S.M. Cunningham, and J.J. Volenec. 2003. Root physiology of less fall dormant, winter hardy alfalfa selections. Crop Sci. 43:1441-1447.

Hekneby, M., M.C., and M. Sánchez-Díaz. 2006. Frost resistance and biochemical changes during cold acclimation in different annual legumes. Environmental and Experimental Botany. 55:305-314.

Hekneby, M., M.C. Antolin, and M. Sanchez-Diaz. 2001. Cold response of annual mediterranean pasture legumes. *In* Delgado I. and J. Lloveras (ed.) Quality in lucerne and medics for animal production. Options Mediterraneennes 45:157-161. (Available online at http://ressources.ciheam.org/om/pdf/a45/01600076.pdf) (verified 04 Sept. 2008).

Hiltbrunner, J., M. Liedgens, L. Bloch, P. Stamp, and B. Streit. 2007. Legume cover crops as living mulches for winter wheat: Components of biomass and the control of weeds. European Journal of Agronomy 26:21-29.

Ipson, R. B. 1991. Thirty years of Wisconsin alfalfa variety trials. M. S. thesis. University of Wisconsin-Madison. Madison, WI.

Julier, B., T. Huguet, F. Chardon, R. Ayadi, J.-B. Pierre, J.-M. Prosperi, P. Barre, and C. Huyghe. 2007. Identification of quantitative trait loci influencing aerial morphogenesis in the model legume *Medicago truncatula*. TAG Theoretical and Applied Genetics 114:1391-1406.

Lesins, K.A., and I. Lesins. 1979. Genus *Medicago* (Leguminosae): a taxogenetic study. Dr. W. Junk bv publishers, The Hague, The Netherlands.

O'Neill, N.R., and G.R. Bauchan. 2000. Sources of resistance in the annual medic core collection. Plant Dis. 84:261-267.

May, 2004. From models to crops:Integrated *Medicago* genomics for alfalfa improvement. p 325-332. *In* A. Hopkins, Z. Wang, and M. Sledge (eds.) Molecular breeding of forage and turf. Kluwer Academic Publishers, Amsterdam.

Moreau, D., C. Salon, and N. Munier-Jolain. 2006. Using a standard framework for the phenotypic analysis of *Medicago truncatula*: an effective method for characterizing the plant material used for functional genomics approaches. Plant, Cell & Environment. 29:1087-1098.

Riday, H., and E.C. Brummer. 2002. Heterosis of Agronomic Traits in alfalfa. Crop Sci 42:1081-1087.

Roe, B., and D. Kupfer. 2004. Sequencing gene rich regions of *Medicago truncatula*, a model legume. p. 333-344. *In In* A. Hopkins, Z. Wang, and M. Sledge (eds.) Molecular breeding of forage and turf. Kluwer Academic Publishers, Amsterdam.

Penmetsa, R.V.and D.R. Cook. 2000. Production and characterization of diverse developmental mutants of *Medicago truncatula.* Plant Physiol. 123:1387-1398.

Sánchez-Díaz, M., M. Hekneby, and M. Antolín. 2000. Cold tolerance of forage legumes growing in controlled continental mediterranean conditions. Cahiers Options Méditerranéennes. 45:265-270.

Schwab, P.M., D.K. Barnes, C.C. Sheaffer, and P.H. Li. 1996. Factors affecting a laboratory evaluation of alfalfa cold tolerance. Crop Sci 36:318-324.

Sheaffer, C.C. 1998. Annual medics in the midwest. p. 14. *In* Proc. 36th. N. Am. Alfalfa Imp. Conf., Bozeman, MT., 2-6 Aug. 1998.

Sheaffer, C.C., D.K. Barnes, D.D. Warnes, W.E. Lueschen, H.J. Ford, and D.R. Swanson. 1992. Seeding-year cutting affects winter survival and its association with fall growth score in alfalfa. Crop Sci. 32:225-231.

Shrestha, A., O. Hesterman, J. Squire, J. Fisk, and C. Sheaffer. 1998. Annual medics and Berseem clover as emergency forages. Agron J. 90: 197-201.

Sultan, K., G. Gintzburger, M. Obaton, C. Robin, H. Touchane, and A. Guckert. 2001. Growth and nitrogen fixation of annual *Medicago*-Rhizobium associations during winter in mediterranean region. Eur. J. Agron. 5:221-229.

Swinnen, J., J.A. Van Veen, and R. Merckx. 1995. Carbon fluxes in the rhizosphere of winter wheat and spring barley with conventional vs integrated farming. Soil Biol. Biochem. 27:811-820.

Taylor, G. B. 1996. Effect of the environment in which seeds are grown and softened on the incidence of autumn seed softening in two species of annual medics. Aust. J. Agricult. Res. 47:141-159.

Teuber, L.R., K.L. Taggard, L.K. Gibbs, M.H. McCaslin, M.A. Peterson, and D.K. Barnes. 1998. Fall dormancy. *In* Standard tests to characterize alfalfa cultivars. 3rd ed. (Amended 1998). N. Am. Alfalfa Imp. Conf., Beltsville, MD. (Available online at http://www.naaic.org/stdtests/Dormancy2.html.) (verified 02 Sept. 2008).

Thoquet, P., M. Ghérardi, E. Journet, A. Kereszt, J. Ané, J. Prosperi, and T. Huguet. 2004. The molecular genetic linkage map of the model legume *Medicago truncatula*: an essential tool for comparative legume genomics and the isolation of agronomically important genes. BMC Plant Biol. 2:1.

Veatch, M. E., S.E. Smith, and G. Vandemark. 2004. Shoot biomass production among accessions of *M. truncatula* exposed to NaCl. Crop Sci. 44: 1008-1013.

Walsh, M.J., R.H. Delaney, R.W. Grosse and J.M. Krall. 2001. Performance of annual medic species (*Medicago* spp.) in Southeastern Wyoming . Agron. J. 93:1249-1256.

Zhu, Y., C. C. Sheaffer and D. K. Barnes. 1996. Forage yield and quality of six annual *Medicago* species in the North-Central USA. Agron. J. 88:955-960.

Zhu, Y., C. Sheaffer, M. Russelle, and C. Vance. 1998. Dry matter accumulation and dinitrogen fixation of annual *Medicago* species. Agronomy Journal 90:103-108.

CHAPTER 2. GROWTH VARIATION AMONG *MEDICAGO TRUNCATULA* ACCESSIONS UNDER SIMULATED AUTUMN CONDITIONS AND THEIR COMPARISON WITH ALFALFA

An article to be submitted to Crop Science

Babita Thapa, E. C. Brummer and Allen Knapp

Abstract

Annual medics have potential as a short-season annual crop to supply forage when traditional supplies are inadequate and as a model crop for studying the genetics of legumes. Assessing growth variation among *M. truncatula* under autumn conditions is an important first step for improving *M. truncatula* yield and identifying potential as a winter annual forage. Our objective was to identify the variation in dry matter accumulation in roots and shoots of *M. truncatula* accessions under simulated autumn (SAC) and constant growth conditions (CGC). Thirty-five *M. truncatula* accessions chosen for high biomass and winter vigor from various areas of collection/origin, and five fall dormancy checks of alfalfa were used for this study. Seeds were planted in the greenhouse and four weeks later seedlings were transferred to growth chambers under SAC and CGC growth conditions. Root dry weight and shoot dry weight were recorded after the treatment period. The time to first flower of the genotypes was also recorded. Genotypes differed for dry weights and first flowering. Root and shoot dry weights of the accessions were higher under modulated autumn conditions than under constant growth temperature. Accessions grown at constant temperature (25°C) flowered earlier compared

to simulated fall conditions. The geographic origin of the accessions used in this study did not appear to indicate growth traits but to time first flower, in some instances. The results revealed the presence of exploitable yield variation among *M. truncatula* accessions. *M. truncatula* has potential as annual forage for harvest in fall. The differential growth performances observed in some of the accessions may also provide interesting materials for future genetic studies.

Introduction

Medicago truncatula Gaertn., barrel medic, is an annual relative of alfalfa (*Medicago sativa* L.) originating in the Mediterranean basin (Lesins and Lesins, 1979). It is one of several annual *Medicago* species, collectively denoted "medics," that are cultivated to a limited extent as a smother crop for weed suppression, green manure crop, cover crop in row crop production, or short-season forage crop (O'Neill and Bauchan, 2000). Medic forage is higher in crude protein and lower in fiber than alfalfa (Sheaffer, 1998). Although *M. truncatula* has less agronomic value than alfalfa, its small genome and short generation time makes it useful as a model crop for legume genomics (Gallardo et al. 2003). Currently, the euchromatic gene space is being sequenced (Roe and Kupfer, 2004) and a host of genomics tools have been developed and are available, some of which - e.g., molecular markers - are directly applicable to alfalfa improvement (Eujayl et al., 2004).

Two traits of importance for commercial production of *M. truncatula* are biomass yield and cold (frost) tolerance. Medics generally produce less dry matter when grown at cool temperatures (10°C) compared to those grown at 20°C, but among the annual medic species evaluated, *M. truncatula* var. Paraggio showed no significant change in total dry

matter and was the most cold tolerant (Heckney et al., 2001). In a later experiment, Paraggio actually had higher stem and root dry matter yield under the lower temperature condition (Antolin et al., 2005). The highest yielding medic cultivars had an extended period of vegetative growth (Walsh et al., 2001), indicating that flowering time affects biomass production. Similarly, in southeastern Wyoming, *M. truncatula* ecotypes were less affected by seasonal influences and maintained higher plant densities than other medic species (Walsh et al., 2001). Temperature and photoperiod affect flowering time in annual medics (Clarkson and Russell, 1975). Generally, *M. truncatula* responds to photoperiod and temperature by flowering earlier under lengthening photoperiods and increasing temperatures (such as occurs during spring) and by flowering later under the opposite conditions, which occur in autumn (Clarkson and Russell, 1975).

Improved forage yield, especially in late summer and autumn, and cold tolerance for winter survival, are also breeding goals for alfalfa, suggesting that *M. truncatula* could be used as a model to help dissect the genetic control of these two traits. The genetic control of late season biomass yield and cold tolerance are intertwined, and simultaneously improving both traits is difficult (Busbice and Wilsie, 1965; Sheaffer et al., 1992, Brummer et. al., 2004). Falling temperatures and shortening photoperiod during the autumn induce cold acclimation in alfalfa plants, thereby increasing their freezing tolerance but concurrently slowing their rate of biomass accumulation (Castonguay et al., 2006). Cold acclimating alfalfa cultivars are considered to be “fall dormant” and typically have higher winter survival but lower forage dry matter yield than non-cold acclimating (aka, “nondormant”) cultivars (Brummer et al., 2000).

Using the annual *M. truncatula* as a model system to study the genetic control of these traits in alfalfa biology faces some hurdles. Most obviously, annual plants do not have overwintering structures, like the alfalfa crown, that need to be maintained under cold temperatures. Similarly, the root-shoot ratios of annual plants are typically smaller than for perennials, which expend more photosynthate on the development of roots to help aid winter survival. Thus, because growth and dry matter allocation patterns among vegetative organs differ in annual and perennial life forms (Jackson and Roy, 1986), we expect that *M. truncatula* and alfalfa may show somewhat different responses to the autumn environment. The cold acclimation response and resulting fall dormancy in annual medics are not known.

We hypothesized that exploitable genetic variation for biomass production is present among the *M. truncatula* accessions and that the growth response of *M. truncatula* to simulated autumn conditions may mirror those seen in alfalfa sufficiently to use the former as a model for the latter. The objective of this paper was to test these hypotheses by evaluating a diverse set of *M. truncatula* accessions and several alfalfa check cultivars under constant and simulated autumn growth conditions in controlled environmental chambers.

Materials and Methods

Seed of *M. truncatula* accessions were obtained from the United States Department of Agriculture National Plant Germplasm System (NPGS), Western Plant Introduction Center, Pullman. Among the 324 accessions available at the station, 33 were selected that covered a range in winter vigor and biomass yield based on the data in the Germplasm

Resources Information Network (GRIN) (Table 1). Most of the accessions were collections that derived from the area of origin for the species, except PI 197341 and PI 442892, which are cultivars from Australia. In addition, seeds of the two breeding lines, DZA 31-16 and Jemalong-6, were obtained from INRA (The National Institute for Agricultural Research, Mauguio, France). Seed of five *M. sativa* cultivars, which serve as checks in the standard evaluation protocol for fall dormancy (Teuber, et al., 1998), were also obtained from the NPGS.

Seeds were scarified with medium grain sandpaper one day before seeding. Six 16-cm tall plastic pots (≈ 2500 cm^3) containing Sunshine Professional growing mix (SB300 Universal Mix, Sun Gro Horticulture Inc., Bellevue, WA, USA) were each planted with fifteen seeds for each accession. The pots were placed in the greenhouse under 25/20 ± 2°C day/night temperature with a 16 h photoperiod and watered daily. After 4 wk, the number of seedlings that emerged in each pot was counted and for one seedling per pot, stem length, fresh weight and dry weight was measured. All pots were then thinned to 3 seedlings per pot.

At this point, the pots were assigned to a growth condition treatment and moved into one of two controlled environment chambers. Each growth chamber had 3 replications of each accession arranged in a randomized complete block design. One of the two chambers was set at constant temperature of 25/20°C day/night with 14 h of light (constant growth condition treatment, CGC). The temperature and the photoperiod of the other chamber was changed on a weekly basis to simulate the progressive temperatures and photoperiods experienced in autumn in central Iowa, USA (simulated autumn growth condition treatment, SAC). The conditions used were the 20-yr mean temperatures and

the photoperiod recorded in Ames Iowa. This treatment began with 24/16°C day/night temperature and 12 h 57 min photoperiod in the first week and decreased to 16/3°C day/night temperature and 11 h 55 min photoperiod at the last week of the treatment. Temperature and photoperiod were adjusted weekly. Growth chamber temperatures were monitored using a HOBO logger (Onset Inc. Pocasset, MA, USA). Light intensity at the top of the plant canopy in both chambers was routinely monitored by a quantum sensor (LI-185, Li-COR Inc., Lincoln, NE, USA) and was maintained at 400 μ mol m^{-2} s^{-1} Photosynthetic Photon Flux density (PPFD) by raising the light bank appropriately as plants grew. Plants were fertilized with a 1:100 ratio of Miracle Grow [15:30:15 N:P:K, 33μL L^{-1} Ca, 13 μL L^{-1} Mg, (The Scotts Miracle-Gro Company, Marysville, OH, USA)] water, with 250 ml added per pot every other week. Plants were watered daily.

After seven weeks of the SAC and CGC treatments, pots were removed from the growth chambers. Plants were removed from the pots and roots carefully washed to remove soil particles. Roots and shoots were separated and the total fresh root and shoot weights per pot were measured. The root and the shoot samples were dried at 60°C for 72 h and weighed. Total dry matter yield per pot was recorded and yield on a per plant basis was computed by dividing the yield by the number of plants per pot (although most pots had three plants, in a few instances, one or two plants died during the experiment).

Prior to harvest, flowering of the accessions was recorded on a weekly basis using a scale of 1 to 8, with 1 for the accessions that began flowering in the first week and 8 for those that did not flower during the course of the experiment. Stem length (SL) was measured on an individual plant at the last week of the treatments by measuring length of the first primary branch in *M. truncatula* accessions, and from the soil surface to the top

of the canopy in alfalfa in a manner analogous to the fall dormancy standard test. The entire experiment was repeated twice, as blocks, with the temperature/photoperiod regimes switched between growth chambers.

For statistical analysis, the genotype and growth conditions were considered to be fixed effects, while blocks and replicates were considered to be random. An analysis of variance was conducted for each trait, with the sources of variation being growth condition, genotype and their interaction using PROC GLM of the SAS (9.1) statistical software package (SAS Institute, Inc., 2002). Significance of each source was determined by an *F*-test. Comparisons were made between alfalfa and *M. truncatula* using a single degree of freedom contrast. Analyses were performed separately for alfalfa cultivars and *M. truncatula* accessions for purposes of comparing means among entries and for correlations among traits. Differences between the treatment means were separated by Fisher's protected least significance (LSD) test at the 5% probability level. Based on individual accession means, the Proc CORR procedure was used to calculate phenotypic correlations. Excluding the Australian cultivars, accessions were placed into East and West groups depending on their origin (accessions ≤9° E longitude were considered West and ≥10° E as East) and the groups were contrasted for the measured traits.

In order to assess relationships among the 35 *M. truncatul*a accessions for these quantitative traits, a principal component analysis (PCA) was conducted including 12 quantitative traits (root dry weight, shoot dry weight, total dry weight, stem length, root:shoot ratio, and time to first flower each measured under constant and simulated autumn conditions) to examine relationships among the accessions. The PCA output was used to develop a distance matrix among the accessions which could subsequently be

used in a cluster analysis to visualize the relationships among accessions. The distance among accessions was developed using PROC Distance by weighting each principal component by the proportion of variance each explained. Ward's method of hierarchical cluster analysis was used to produce clusters, which were visualized using PROC Tree. Major clusters of the dendrogram were analyzed using a one-way analysis using PROC ANOVA for their characterization based on the 12 quantitative traits. Throughout the results and discussion, statistical significance was assessed at the 5% probability level, unless noted otherwise.

Results

The growth condition by accession interaction was significant for root dry weight, shoot dry weight, total dry weight, root-shoot ratio and time to first flower, and consequently, we analyzed each growth treatment separately. The interaction was not significant for stem length. All traits except root-shoot ratio differed between the two growth conditions (data not shown). Overall, root and shoot dry weights increased under SAC compared to CGC while root-shoot ratio remained the same (Table 2). *M. truncatul*a plants grown under CGC had longer stems and flowered earlier than under SAC. The *M. truncatula* accessions differed for all traits (data not shown), with a wide range of variation observed (Table 2).

Alfalfa cultivars differed for all the growth traits (data not shown), but the effect of growth condition was only observed for stem length and time to first flower. The most dormant cv. Vernal had the largest reduction (7 cm, P= 0.02) in stem length under SAC when compared to CGC and UC 1887 which had the smallest reduction (1 cm, P=0.49).

Over all, alfalfa grown under CGC flowered earlier and had longer stems than when grown under SAC, similar to the results observed for *M. truncatula* (Table 2).

Within each growth condition, root and shoot dry weight were positively correlated with each other and each was highly correlated with total dry matter as expected (Table 3). Stem length was positively correlated with shoot dry weight in both of the growth conditions while it was also correlated with total dry weight in CGC. Under SAC, root dry weight and root:shoot ratio were positively correlated with the time to first flower; neither correlation was noted under CGC.

We compared *M. truncatula* accessions based on their latitude and longitude of origin and also contrasted *M. truncatula* with *M. sativa* for each of the quantitative traits (Table 4) using the data from both the growth conditions. Accessions from west of 9° E longitude produced more shoot dry weight and longer stems and flowered earlier than those accessions to the east. An almost 2 wk delay in the first flowering was observed in accessions originating from ≥40° N compared to <40° N latitude. However, variation in dry weight was not associated with the continent from which the *M. truncatula* accessions derived. Accessions from European countries flowered later than the African or Australian accessions.

M. truncatula and *M. sativa* differed for all traits, especially shoot dry weight and time to first flower (Table 4). *M. truncatula* accessions had higher shoot and total dry matter after 11 wk and developed longer stems, while root dry weight and root:shoot ratio were higher for alfalfa cultivars. Alfalfa cultivars, on average, flowered later than *M. truncatula* accessions; in fact, under simulated autumn conditions, none of the alfalfa cultivars flowered during the study period (Table 2).

Relationships among *M. truncatula* accessions were evaluated using principal components analysis of the twelve quantitative traits measured under the two treatments. The entire variance was accounted for by nine principle components (PCs), with the first three PCs cumulatively accounted for 73% of the total variance (Table 5). PC1 explained 42% of total variation among accessions and primarily included dry weight and root:shoot ratio variables (Table 6). Twenty percent of the total variation was associated with differences in shoot growth among accessions as indicated by positive loading for shoot dry weight and stem length for both treatments in PC2. Variation in stem length among the accessions and differences in flowering time between the two treatments (negative loading in CGC and positive in SAC) accounted for 11% of the variation in PC3. Two dimensional plots did not show any clear distribution of accessions, as has been shown for PC1 vs. PC2 in Fig. 1. Therefore, cluster analysis was performed to further characterize the accessions using the nine PCs.

In the cluster analysis, accessions were clearly grouped into four main clusters (Fig. 2). The first cluster had 11 accessions originated from various countries (35 to 56° N lat) that produced higher dry weights, higher root:shoot ratio but was late to first flower compared to rest of the accessions in other clusters (Table 6) in both the growth conditions. The second cluster was composed of six accessions of which only three were originated from the Mediterranean region. In contrast to the first cluster, these accessions produced low dry weights and flowered early regardless of the growth conditions. Accessions in cluster three produced lower total dry weights than the first cluster in both growth conditions due to reduced root dry weights. While the accessions in the first and the third cluster flowered at the same time in SAC, the later flowered earlier in CGC. The

fourth cluster produced similar dry weights and stem length as the third cluster except for flowering time and RS ratio. These two clusters differed in their response to the treatments for the time to first flower. Accession in the third cluster delayed flowering in SAC compared to CGC while fourth cluster accessions did not show such changes.

Discussion

M. truncatula accessions vary substantially for growth characteristics and time to first flower when grown as seedlings under either constant growth or simulated autumn conditions. This is not surprising as annual medics are known to be highly adaptive to diverse temperatures and locations (Crawford et al., 1989). The majority of the accessions used in the study were wild collections whose diverse genetic backgrounds likely provide the differential environmental adaptations to explain the observed growth differences. The accessions grown under simulated autumn conditions resulted in higher dry weights than those grown under constant growth conditions. This result supports the previous observation in *M. truncatula* cv. Paraggio, where plants grown at 10°C obtained comparable leaf dry weight but higher root and stem dry weights compared to 20°C through an efficient photosynthetic acclimation (Antolin et al., 2005). In the Mediterranean climate, annual legumes grow during the cool season when rain is possible, avoiding the dry summer months (Sultan et al. 2001) and therefore they may have developed differential abilities in cold tolerance in the process of gradual adaptation to the low temperatures. Variation in shoot biomass and dry weights among 21 *M. truncatula* accessions have been reported in an assessment of salt tolerance (Veatch et al. 2004).

Differences in flowering time between far northern to northern, and also between eastern to western originated accessions (Table 4) indicate the influence of environmental factors for the transition from vegetative to flowering stage and adaptation to different native environments. Flowering in *M. truncatula* is accelerated by vernalization, long days, and high temperature, and interactions among these processes can further modulate flowering time (Clarkson and Russell, 1975). The critical photoperiod to stimulate flowering is between 12-18 h. A photoperiod greater than 18 h stimulated flowering 61 d earlier in Jemalong than 12 h (Clarkson and Russell, 1975). We observed a week delay on average in the first flowering under SAC compared to CGC plants. Delay in flowering increased the root matter of the plants and favored the partitioning of dry matter to roots over shoots and increase root:shoot ratios, as indicated by its positive correlation with the two traits in SAC plants. Dry matter production in annual medics is related to periods of growth and development where high yielding cultivars show an extended period of vegetative growth (Walsh et al., 2001). The greatest transfer of net fixed carbon in roots occurs at the vegetative stage (Swinnen et al., 1995). Therefore, a longer vegetative period enables plants to produce higher root dry weights than a short period (Rees et al., 2005).

We showed that *M. truncatula* accumulated more dry matter than perennial *M. sativa*. The higher root dry weights and root-to-shoot ratio of *M. sativa* compared to *M. truncatula* may be associated with the differences in their resource allocation pattern associated with different life forms. Theoretical models for growth and development predict an instant switch from vegetative to flowering growth in annuals (King and Roughgarden, 1983) while perennials show a complex pattern between growth stages

involving plant age and resource status (Kozlowski, 1992). Growth analysis based on seven congeneric annual and perennial grass species revealed that annuals had higher relative growth rates, relative leaf production ratesand unit leaf area rates than perennials (Garnier, 1992). Annual plants allocate a higher amount of biomass to shoots to sustain high growth (Jackson and Roy, 1986). Perennials accumulate high proportions of biomass in roots to support the tall shoots, survive when carbon export is minimal, support regrowth in the subsequent year and tolerate a wide fluctuation of soil temperatures (Essenstate, 1997).

The PCA was successful in isolating groups from the total accessions based on 12 quantitative traits. *M. truncatula* accessions grouped into four major clusters in this study, suggesting a strategy for selecting a subset of accessions for further study (Fig. 2). The clustering pattern corresponded more closely to average growth traits and flowering date (Table 6), as indicated by the allotment of accessions from similar locations into different clusters (e.g. accessions from Morocco where located in I, III and IV clusters). While other evidence proved that quantitative traits in some *M. truncatula* accessions are associated with their adaptation to their ecological conditions as accessions from similar geographical locations are grouped into the same cluster (e.g. cluster I included far northern originated accessions). Our results are somewhat consistent with a study of 350 natural populations collected around the Mediterranean basin and 231 introductions from Australia wherein the authors identified four main clusters (Ronfort et al., 2006). The genotypes in their study revealed a large amount of genetic diversity and allelic variation. In addition, clustering of the accessions also occurred based on ability to alter the phenotypic traits in response to growth conditions evident by comparison between

clusters I and III that flowered at different times in CGC flowered at the same time in SAC. Both the clusters showed acclimation behavior with the reduction in stem length but the accessions in the first did it without the change in flowering time and lower dry weights reduction than cluster II accessions. These results may imply that *M. truncatula* accessions vary in their ability to avoid cold temperature stress and cold tolerance.

In this study, alfalfa checks demonstrated fall dormancy behavior as indicated by reduced stem length in SAC compared to CGC and ranked according to their dormancy rating, except no clear distinction was seen between ABI 700 and Pierce. Similarly, most of the *M. truncatula* accessions had reduced stem length in SAC condition. Additionally, both *M. truncatula* and *M. sativa* showed a delay in flowering time in SAC. These results may suggest that *M. truncatula* may respond to fall weather conditions and undergo gradual physiological changes to improve cold tolerance, similar to the autumn dormancy acclimation response commonly observed in winter hardy alfalfa cultivars.

A number of accessions that displayed unique phenotypic characteristics in the study may be of interest for the future studies. A few accessions, PI 464815 (Turkey), W6 6096 (Germany), W6 5018 (Italy), PI 577434 (Tunisia) and W6 6076 (Morocco) did not flower under either growth condition suggesting they have a long period of vegetative growth which was not applied in this study. In contrast, W6 5983 (Cyprus) was the earliest to flower regardless of growth conditions. These accessions could be useful for understanding genetic and environmental control of flowering of *M. truncatula*. Accessions PI 516931 and W6 5018 had the most erect plant type with a tall main axis. Jemalong-6 displayed the most dormant phenotype upon simulated autumn treatment with a greatly reduced main axis, condensed internodes with many tiny leaves around the

axis and had pigmentation on leaves, unlike the rest of the accessions. Accession W6 6143 (Algeria) had profuse root growth and a high root:shoot ratio. Other accessions, including W6 4980 (France), PI 319051 (Spain), PI 442892 (Australia), W6 6029 (Italy) and W6 5018 (Italy), did not reduce stem length in SAC compared to CGC. These results suggest that accessions may show differences for cold tolerance and for environmental signals to cold acclimate.

In conclusion, our results revealed the existence of a high level of genetic diversity in dry weights and time to first flower among *M. truncatula* accessions, and among accessions within the same geographical location. Time to first flower was the only trait that differed between contrasting locations and between the growth conditions which may suggest that *M. truncatula* has wide adaptation abilities to diverse environments. The annual *M. truncatula* and perennial *M. sativa* showed different growth behavior based on the traits that may be associated with their growth habit, but nevertheless, displayed similar responses (e.g. delay in time to first flowering and reduction in stem length) in simulated autumn conditions, indicating *M. truncatula* accessions may undergo cold acclimatization in autumn weather conditions similar to alfalfa and may have considerable cold tolerance. This was further confirmed by high dry weights observed in simulated autumn conditions used in the study. Overall, it appears that *M. truncatula* has potential to help explain genetic differences in autumn yield and cold tolerance of other legumes, including alfalfa.

References

Antolin, M.C., M. Hekneby, and M. Sanchez-Diaz. 2005. Contrasting responses of photosynthesis at low temperatures in different annual legume species. Photosynthetica 43:65-74.

Brummer, E.C. 2004. Applying genomics to alfalfa breeding program. Crop Sci. 44:1904-1907.

Brummer, E.C., M.M. Shah, and D. Luth. 2000. Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. Crop Sci. 40:971-977.

Busbice, T.H., and C.P. Wilsie. 1965. Fall growth, winterhardiness, recovery after cutting and wilt resistance in F2 progenies of Vernal X DuPuits alfalfa crosses. Crop Sci. 5:429-432.

Castonguay, Y., S. Laberge, E.C. Brummer, and J. Volenec. 2006. Alfalfa winter hardiness: A research retrospective and integrated perspective. Adv. Agron. 90:203-265.

Clarkson, N.M., and J.S. Russell. 1975. Flowering responses to vernalization and photoperiod in annual medics (*Medicago* spp.). Aus. J. Agric. Res. 26:831-838.

Crawford, J.E., A.W.H. Lake, and K.G. Boyce. 1989. Breeding annual *Medicago* species for semiarid conditions in southern Australia. Adv. agron. 42:399-437.

Eissenstat, D.M. 1997. Trade-offs in root form and function. p. 173-196. *In* Louise E. Jackson (ed.) Ecology in Agriculture. Academic Press.

Eujayl, I., M. Sledge, L. Wang, G. May, K. Chekhovskiy, J. Zwonitzer, and M. Mian. 2004. *Medicago truncatula* EST-SSRs reveal cross-species genetic markers for *Medicago* spp. Theor. Appl. Genet. 108:414-422.

Gallardo, K., C. Le Signor, J. Vandekerckhove, R.D. Thompson, and J. Burstin. 2003. Proteomics of *Medicago truncatula* seed development establishes the time frame of diverse metabolic processes related to reserve accumulation. Plant Physiol. 133:664-682.

Garnier, E. 1992. Growth analysis of congeneric annual and perennial grass species. J. Ecol. 80:665-675.

Hekneby, M., M.C. Antolin, and M. Sanchez-Diaz. 2001. Cold response of annual mediterranean pasture legumes. *In* Delgado I. and J. Lloveras (ed.) Quality in lucerne and medics for animal production. Options Mediterraneennes 45:157-161. (Available online at http://ressources.ciheam.org/om/pdf/a45/01600076.pdf) (verified 04 Sept. 2008).

Jackson, L.E., and J. Roy. 1986. Growth patterns of mediterranean annual and perennial grasses under simulated rainfall regimes of southern France California. Oecol. Plant. 7:191-212.

King, D., and J. Roughgarden. 1983. Energy allocation patterns of the California grassland annuals *Plantago erecta* and *Clarkia rubicunda*. Ecology (Durham) 64:16-24.

Kozlowski, J. 1992. Optimal allocation of resources to growth and reproduction: Implications for age and size at maturity. Trends Ecol. Evol. 6:15-19.

Lesins, K.A., and I. Lesins. 1979. Genus *Medicago* (Leguminosae): a taxogenetic study. Dr. W. Junk bv publishers, The Hague, The Netherlands.

O'Neill, N.R., and G.R. Bauchan. 2000. Sources of resistance in the annual medic core collection. Plant Dis. 84:261-267.

Rees, R.M., I.J. Bingham, J.A. Baddeley, and C.A. Watson. 2005. The role of plants and land management in sequestering soil carbon in temperate arable and grassland ecosystems. Mechanisms and regulation of organic matter stabilisation in soils. Geoderma 128:130-154.

Roe, B., and D. Kupfer. 2004. Sequencing gene rich regions of *Medicago truncatula*, a model legume. p. 333. *In* Molecular breeding of forage and turf. Kluwer Academic Publishers, Amsterdam.

Ronfort, J., T. Bataillon, S. Santoni, M. Delalande, J. David, and J.-M. Prosperi. 2006. Microsatellite diversity and broad scale geographic structure in a model legume: building a set of nested core collection for studying naturally occurring variation in Medicago truncatula. BMC Plant Biology 6:28.

Sheaffer, C.C. 1998. Annual medics in the midwest. p. 14. *In* Proc. 36th. N. Am. Alfalfa Imp. Conf., Bozeman, MT., 2-6 Aug. 1998.

Sheaffer, C.C., D.K. Barnes, D.D. Warnes, E. Lueschen, H.J. Ford, and D.R. Swanson 1992. Seeding-Year cutting affects winter survival and its association with fall growth score in alfalfa. Crop Sci. 32:225-231.

Sultan, K., G. Gintzburger, M. Obaton, C. Robin, H. Touchane, and A. Guckert. 2001. Growth and nitrogen fixation of annual *Medicago*-Rhizobium associations during winter in mediterranean region. Eur. J. Agron. 5:221-229.

Swinnen, J., J.A. Van Veen, and R. Merckx. 1995. Carbon fluxes in the rhizosphere of winter wheat and spring barley with conventional vs integrated farming. Soil Biol. Biochem. 27:811-820.

Teuber, L.R., K.L. Taggard, L.K. Gibbs, M.H. McCaslin, M.A. Peterson, and D.K. Barnes. 1998. Fall dormancy. *In* Standard tests to characterize alfalfa cultivars. 3rd ed. (Amended 1998). N. Am. Alfalfa Imp. Conf., Beltsville, MD. (Available online at http://www.naaic.org/stdtests/Dormancy2.html.) (verified 02 Sept. 2008).

Veatch, M. E., S.E. Smith, and G. Vandemark. 2004. Shoot biomass production among accessions of *M. truncatula* exposed to NaCl. Crop Sci. 44: 1008-1013.

Walsh, M.J., R.H. Delaney, R.W. Grosse and J.M. Krall. 2001. Performance of annual medic species (*Medicago* spp.) in Southeastern Wyoming . Agron. J. 93:1249-1256.

Table 1.Country of origin, latitude, and longitude of the *M. truncatula* accessions and alfalfa dormancy checks used in the study.

Entry No.	Accession	Origin	Latitude		Longitude	
Medicago truncatula						
1	W6 5018	Italy	41°28'	N	013°11'	E
2	PI 197361	Australia†	35°16'	S	139°27'	E
3	PI 516931	Morocco	33°46'	N	007°15'	W
4	PI 535648	Tunisia		N		W
5	W6 4996	Greece	38°00'	N	023°44'	E
6	W6 5974	Greece	35°22'	N	025°10'	E
7	W6 5983	Cyprus	35°15'	N	033°29'	E
8	W6 5987	Cyprus	34°40'	N	032°56'	E
9	W6 6027	Malta	35°58'	N	014°22'	E
10	W6 6076	Morocco	33°46'	N	006°39'	W
11	W6 6000	France	42°05'	N	009°30'	E
12	W6 6018	Germany†		N		E
13	W6 6029	Italy	41°49'	N	016°12'	E
14	W6 6035	Spain		N		W
15	W6 6048	Tunisia	34°58'	N	008°35'	E
16	PI 197341	Australia†‡		S		E
17	PI 384636	Morocco	32°03'	N	008°29'	W
18	W6 4980	France	42°46'	N	002°42'	E
19	W6 6096	Germany†		N		E
20	W6 6143	Algeria	35°26'	N	001°46'	E
21	W6 6108	Italy	46°02'	N	012°52'	E
22	PI 319051	Spain	41°05'	N	004°19'	W
23	PI 384635	Morocco	34°27'	N	006°29'	W
24	PI 442892	Australia†‡		S		E
25	PI 464815	Turkey		N		E
26	PI 516925	Morocco	33°36'	N	007°05'	W
27	PI 516942	Morocco	32°29'	N	007°12'	W
28	PI239873	Algeria	36°28'	N	003°00'	E
29	PI 239877	Morocco	31°14'	N	008°14'	W
30	PI 577643	Malta		N		E
31	PI 577639	Sweden†	56°18'	N	016°28'	E
32	PI 577434	Tunisia	35°51'	N	009°12'	E
33	PI 517256	Ethiopia†‡		S		E
34	DZA319-16	France				
35	Jemalong -6	France‡				
Alfalfa Checks						
36	ABI 700					
37	Vernal					
38	Pierce					
39	Legend					
40	UC 1887					

† Collections from country not bordering the Mediterranean sea

‡ Entries donated from the country

Table 2. Ranges, means and standard deviations (SD) of growth and flowering traits† of *M. truncatula* accessions and *M. sativa* standard cultivars in simulated autumn (SAC) and constant temperature conditions (CGC). Mean separation was performed separately for *M. truncatula* and *M. sativa* genotypes.

Trait	Simulated autumn condition (SAC)		Constant temperature condition (CGC)	
	Mean ± SD	Range	Mean ± SD	Range
M. truncatula				
RDW, g plant^{-1}	0.69 ± 0.20a‡	0.18-1.17	0.60 ± 0.16b	0.63-1.43
SDW, g plant^{-1}	2.07 ± 0.25a	1.20-2.90	1.50 ± 0.30b	0.60-2.50
TDW, g plant^{-1}	2.74 ± 0.34a	1.50-3.80	2.10 ± 0.39b	0.70-3.90
RSR	0.31 ± 0.11a	0.16-0.56	0.38 ± 0.12a	0.19-0.57
SL, cm	31.40 ± 5.21b	21.00-45.00	36.00 ± 5.40a	30.00-43.00
FF, wk	4.82 ± 1.00b	0.00-5.00	3.67 ± 0.50a	0.00-4.50
M. sativa				
RDW, g plant^{-1}	0.62 ± 0.10a	0.47-0.78	0.75 ± 0.21a	0.34-1.06
SDW, g plant^{-1}	1.07 ± 0.19a	0.70-1.30	1.14 ± 0.21a	0.60-1.20
TDW, g plant^{-1}	1.69 ± 0.19a	1.40-1.90	1.88 ± 0.47a	0.90-2.20
RSR	0.65 ± 0.20a	0.49-0.98	0.78 ± 0.17a	0.51-0.98
SL, cm	29.89 ± 2.10b	24.40-36.00	32.19 ± 2.50a	29.85-37.68
FF, wk	0.00 ± 0.00a	0.00-0.00§	4.00 ± 1.10b	0.00-5.00

†RDW (root dry wt., g plant^{-1}), SDW (shoot dry wt., g plant^{-1}), TDW (total dry wt., g plant^{-1}), RSR (root-to-shoot ratio), SL (stem length, cm) and FF (time to first flower, week).

‡Mean columns of SAC and CGC with same lowercase letters were not significantly different for the corresponding trait based on LSD values (P =0.05).

§no flowering occurred within the treatment period

Table 3. Pearson's correlation coefficients for growth and flowering traits† of 35 *M. truncatula* accessions grown in simulated autumn (SAC) and constant growth conditions (CGC).

Trait	Simulated autumn condition (SAC)					Constant growth condition (CGC)					
	SDW	TDW	RSR	SL	FF	RDW	SDW	TDW	RSR	SL	FF
SAC											
RDW	0.56***	0.81***	0.76***	-0.01	0.35*	0.57***	0.32	0.47**	0.41*	0.01	0.11
SDW		0.93***	-0.07	0.41*	- 0.15	0.54**	0.53**	0.58***	0.18	0.20	0.35*
TDW			0.25	0.17	0.04	0.56**	0.45*	0.56***	0.29	0.13	0.22
RSR				-0.14	0.55***	0.21	0.01	0.21	0.50**	-0.19	-0.08
SL					-0.12	0.13	0.36*	0.24	-0.05	0.62***	0.33
FF						0.19	0.40*	0.25	0.38*	-0.09	0.54**
CGC											
RDW							0.76***	0.91***	0.77***	0.27	0.17
SDW								0.95***	0.27	0.34*	0.14
TDW									0.50**	0.36*	0.16
RSR										-0.09	0.14
SL											-0.01

*Significance at the probability level of 0.05
**Significance at the probability level of 0.01
***Significance at the probability level of 0.001

† RDW (root dry wt., g plant^{-1}), SDW (shoot dry wt., g plant^{-1}), TDW (total dry wt., g plant^{-1}), RSR (root-to-shoot ratio), SL (stem length, cm) and FF (time to first flower, week).

Table 4. Contrasts, among *M. truncatula* accessions based on origin and with *M. sativa* cultivars, their mean squares and significance on growth and flowering traits† of simulated autumn and constant growth conditions.

	Trait					
Contrast	RDW	SDW	TDW	RSR	SL	FF
European Vs African	0.01	0.01	0.01	0.00	16	2.52*
East (≥010°E) Vs West(004°W- <010° E)	0.02	3.32**	3.83*	0.06	894***	56.91***
North <40° Vs North ≥40°	0.08	0.32	0.58	0.01	160	93.25***
Australian Vs other *truncatula*	0.04	0.11	0.27	0.01	72	1.83*
M. sativa Vs *M. truncatula*	0.24*	3.52***	1.85*	1.39*	126*	34.42***

*Significance at the probability level of 0.05.
**Significance at the probability level of 0.01.
***Significance at the probability level of 0.001.

†RDW (root dry wt., g plant^{-1}), SDW (shoot dry wt., g plant^{-1}), TDW (total dry wt., g plant^{-1}), RSR (root-to-shoot ratio), SL (stem length, cm) and FF (time to first flower, week).

Table 5. Eigenvectors and the principal components of 12 quantitative traits in *M. truncatula* accessions at simulated autumn condition (SAC) and constant growth condition (CGC).

Traits†	Principal Component								
	1	2	3	4	5	6	7	8	9
RDW (SAC)	**0.41**	**-0.11**	**-0.17**	**0.09**	**-0.20**	**-0.15**	**-0.00**	**-0.01**	**-0.42**
SDW (SAC)	**0.28**	**0.36**	**-0.37**	**0.04**	**0.06**	**0.28**	**0.24**	**0.11**	**0.40**
TDW (SAC)	**0.37**	**0.21**	**-0.33**	**0.07**	**-0.03**	**0.13**	**0.16**	**0.07**	**0.11**
RSR (SAC)	**0.27**	**-0.41**	**0.09**	**0.14**	**-0.32**	**-0.40**	**-0.13**	**-0.07**	**0.64**
SL(SAC)	**0.05**	**0.41**	**0.37**	**0.46**	**-0.03**	**-0.21**	**-0.19**	**0.62**	**-0.03**
FF (SAC)	**0.17**	**-0.37**	**0.35**	**0.11**	**0.35**	**-0.00**	**0.74**	**0.17**	**-0.31**
RDW (CGC)	**0.41**	**-0.11**	**-0.17**	**0.09**	**-0.20**	**-0.15**	**-0.00**	**-0.18**	**-0.42**
SDW (CGC)	**0.32**	**0.21**	**0.16**	**-0.33**	**0.39**	**-0.27**	**-0.17**	**-0.13**	**0.17**
TDW (CGC)	**0.35**	**0.13**	**0.25**	**-0.38**	**0.27**	**-0.06**	**-0.17**	**-0.05**	**-0.14**
RSR (CGC)	**0.23**	**-0.24**	**0.31**	**-0.32**	**-0.32**	**0.63**	**-0.21**	**0.32**	**0.05**
SL(CGC)	**0.11**	**0.37**	**0.48**	**0.23**	**-0.33**	**0.19**	**0.19**	**-0.61**	**-0.11**
FF (CGC)	**0.15**	**-0.25**	**-0.31**	**0.55**	**0.49**	**0.36**	**-0.41**	**-0.22**	**0.00**

†RDW (root dry wt., g plant^{-1}), SDW (shoot dry wt., g plant^{-1}), TDW (total dry wt., g plant^{-1}), RSR (root-to-shoot ratio), SL (stem length, cm) and FF (time to first flower, week).

Table 6. Characteristics of major clusters based 12 quantitative traits† averaged across accessions within a cluster measured in simulated autumn condition and constant growth condition.

Group	Simulated autumn condition						Constant growth condition					
	SDW	RDW	TDW	SL	RSR	FF	SDW	RDW	TDW	SL	RSR	FF
I	2.43a§	0.96a	3.37a	30.4bc	0.38a	5.9a	1.78a	0.82a	2.61a	36.7a	0.46a	5.2a
II	1.66c	0.38c	2.04c	25.4c	0.22b	2.5b	1.01b	0.32d	1.33c	31.1b	0.31b	1.8d
III	1.87bc	0.73b	2.60bc	31.8ab	0.39a	6.1a	1.4ab	0.61b	2.03b	35.9a	0.43a	3.6b
IV	2.18ab	0.53bc	2.71b	36.3a	0.24b	2.8b	1.61a	0.49c	2.1b	40.2a	0.30b	2.9c

†RDW (root dry wt., g plant^{-1}), SDW (shoot dry wt., g plant^{-1}), TDW (total dry wt., g plant^{-1}), RSR (root-to-shoot ratio), SL (stem length, cm) and FF (time to first flower, week).

§Average values (n=6) in column for a trait with same lowercase letters were not significantly different based on LSD values (P =0.05).

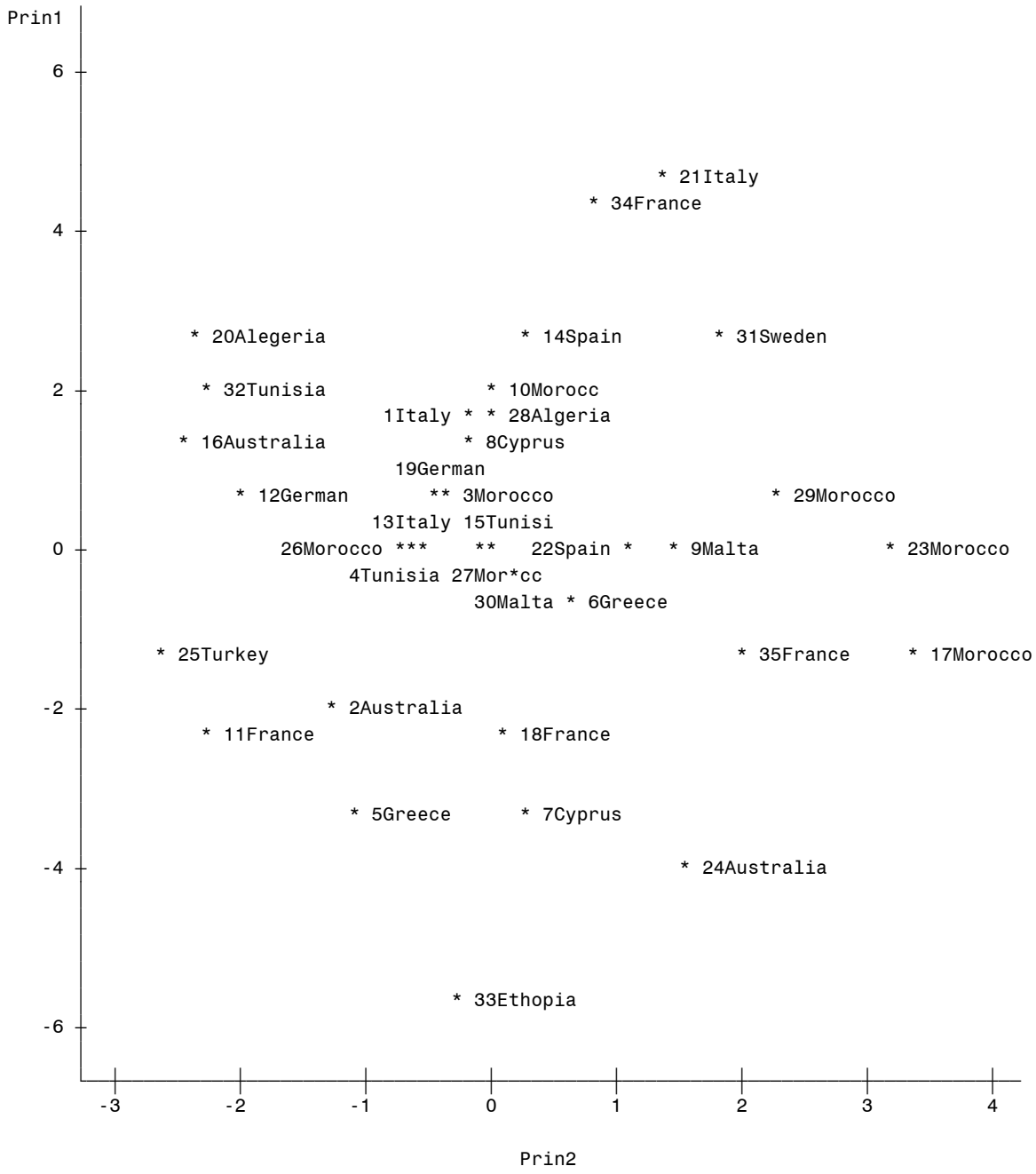


Figure 1. Scatter plot using PCA1 and PCA2 representing 35 *M. truncatula* accessions along with their country of origin.

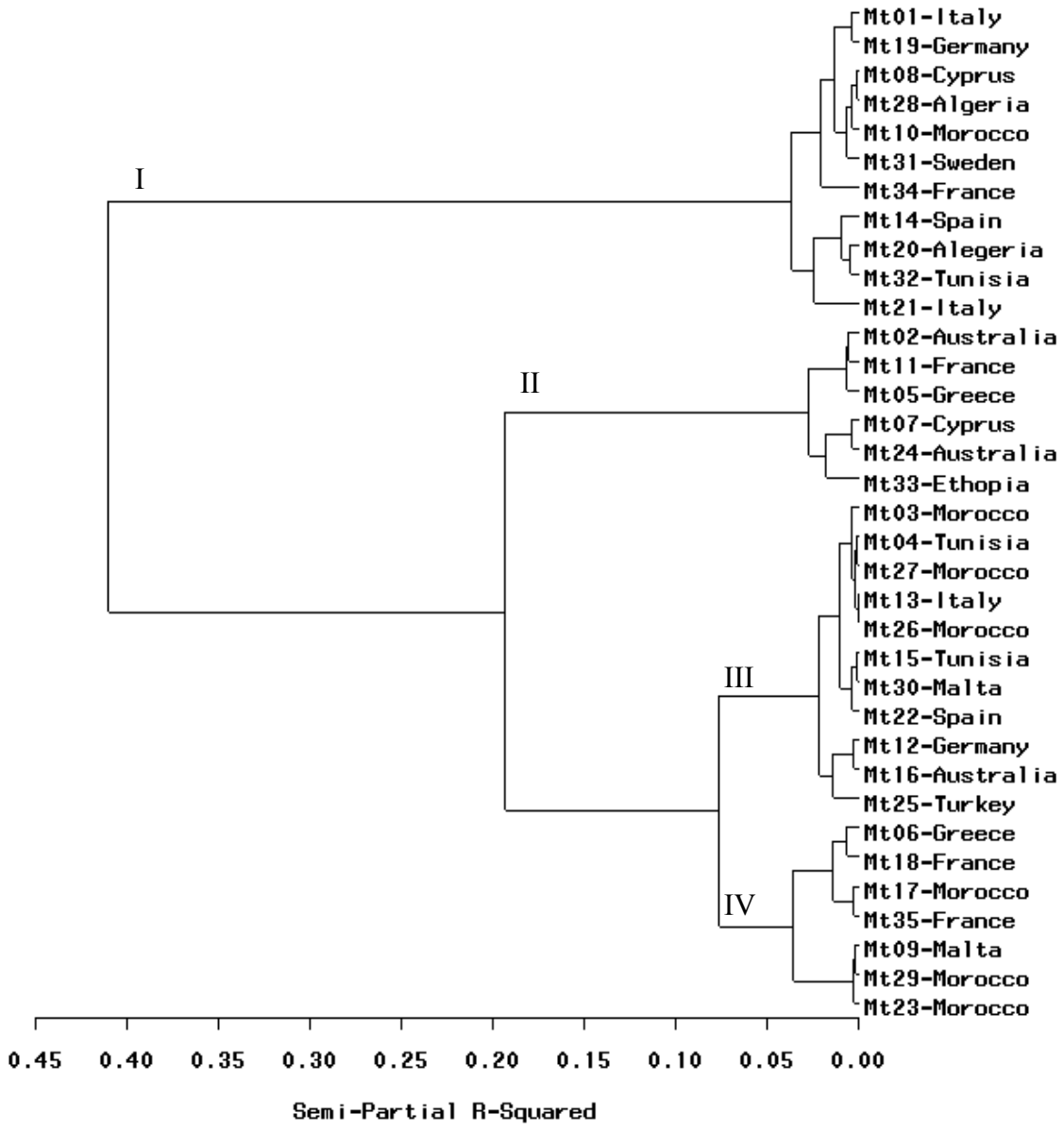


Fig.2. Dendrogram of 35 *M. truncatula* accessions using 9 principal components based on six growth and flowering traits.

CHAPTER 3. APPLYING FREEZING TEST TO QUANTIFY COLD ACCLIMATION IN *MEDICAGO TRUNCATULA* GAERTN.

Babita Thapa[1]
Department of Agronomy, Iowa State University, Ames, IA 50010.

Rajeev Arora
Department of Horticulture, Iowa State University, Ames, IA 50010

Allen D. Knapp
Department of Agronomy, Iowa State University, Ames, IA 50010.

E. Charles Brummer
Department of Crop and Soil Sciences, University of Georgia, Athens, GA 30602

[1]Corresponding author; e-mail: bthapa@iastate.edu
Telephone: 1-515-294-9721
Fax: 1-515-294-5506
Manuscript received: . . . . . .
Manuscript accepted: . . . . . .

ADDITIONAL INDEX WORDS. Barrel medic, ion leakage, freezing tolerance

ABSTRACT. Understanding cold acclimation (CA) is important for concurrently improving autumn yield and winter survival in alfalfa (*Medicago sativa* L.). *Medicago truncatula* Gaertn., an annual relative of alfalfa, could be used to determine genetic bases of CA, if the ability and conditions required for its CA are determined. The major

objective of this study was to develop a laboratory screening procedure to quantify cold acclimation in *M. truncatula*. Two genotypes, Jemalong-6 and W6 5018 were grown in non-acclimation (NA) and three cold acclimation regimes (CA1, CA2, CA3). Cold acclimation was quantified by measuring freezing tolerance (LT_{50}; the freeze temperature at which 50% injury occurred), as estimated by ion leakage (IL) from leaf tissues. Percent injury and LT_{50} were derived from freeze injury data. Cold-acclimated plants had reduced stem length, number of leaves, stem dry weight, leaf dry weight and root dry weight compared to control. Root-to-shoot ratio was higher in cold-acclimated than in control plants. These results indicate the clear initiation of acclimation response in cold-acclimated plants. While 80% injury was induced by -7 °C in NA plants, -20 °C only induced 52% injury in CA3 plants. Average LT_{50} temperatures were -3, -8, -7.8 and ≥ -12.5 °C in NA, CA1, CA2 and CA3 regimes, respectively. Percent injury was lower in Jemalong-6 than in W6 5018 for all cold acclimation regimes. Our results demonstrated the capability of *M. truncatula* to cold acclimate under the controlled CA regimes and the possible use of IL as a rapid laboratory method to quantify cold acclimation.

Introduction

Increased autumn growth is one means of improving yield in alfalfa (*Medicago sativa* L.). However, an inverse relationship between biomass and winter hardiness often complicates the simultaneous improvement of both traits (Brummer, 2004). Alfalfa is known to acclimate as temperatures fall and photoperiod decreases during autumn (McKenzie et al., 1988), and significant advances in understanding the physiological changes that alfalfa undergoes during cold acclimation have been made (Castonguay et

al., 2006). However, alfalfa is a tetrasomic tetraploid, complicating genetic analysis, and outcrossing with inbreeding depression, that prevents the development of inbred lines. Both aspects of alfalfa hinder investigations on the genetic basis of autumn acclimation (also called "fall dormancy"), cold tolerance, and winter survival.

M. truncatula (barrel medic) is an annual legume, closely related to alfalfa, grown as winter forage and green manure crop in Mediterranean climatic zones around the world. In the north-central USA, annual medics can produce high yields of quality forage when grown as short season annual crops for autumn harvest (Zhu et al., 1996). Unlike alfalfa, *M. truncatula* is diploid and self-pollinated, characteristics that make it more tractable for genetic analysis than alfalfa. Although *M. truncatula* does not survive winter conditions in the northern USA, it often encounters and survives frost when cultivated, and therefore, it may offer some clues about cold tolerance in alfalfa.

Numerous biochemical/physiological changes occur during cold acclimation including increases in cold-stress proteins (Arora and Wisniewski, 1994; Arora et al., 1997; Marian et al., 2004); accumulation of cryoprotectants such as proline (Wanner and Junttila, 1999) and sugar (Haagenson et al., 2003; Koster and Lynch, 1992; Patton et al. 2007); and increases in the unsaturated to saturated fatty acid ratio and in phospholipids in the plasma membrane (Palta et. al, 1993). A key function of cold acclimation is to stabilize membranes against freezing-induced cellular dehydration (Thomashow, 1999). Ion leakage results, from decreased membrane integrity of freeze damaged tissues, can be easily measured in the laboratory to determine freezing tolerance of diverse species (Arora and Palta, 1991; Nunes and Smith, 2003; Webb et al., 1994; Welling et al., 2002), including alfalfa (Sulc et. al., 1991). This method is simple, repeatable, and rapid, ensures

freezing stress on intact tissues, and has been used to predict field performance (Teutonico et al., 1993).

M. truncatula cold acclimation has been assessed in several experiments, with contradictory results. The cultivar Paraggio, grown at 10 °C, appeared to acclimate to the cool temperature by exhibiting a two-fold increase in the quantum yield of photosytem II electron transport (Antolín et al., 2005). In contrast, plants exposed to cool conditions (10 °C day/5 °C night) exhibited poor regrowth capacity compared to non-acclimated plants when it was assessed by measuring dry matter production after freezing test. The cold-acclimated plants had decreased sucrose phosphate activity in leaves and with no accumulation of starch in roots, suggesting ineffective cold acclimation process in the species (Hekneby and Sánchez-Díaz, 2006). Brandsæter et al. (2000) evaluated 12 winter legume species hardened at 2 °C for 2 weeks to investigate their potential as overwintering cover crops and reported the poorest frost survival in *M. truncatula*. The lack of consensus among these studies on the ability of *M. truncatula* to exhibit cold acclimation possibly results from the different freezing test procedures employed or the different genotypes used in the studies.

In this experiment, we developed a freezing tolerance assay for *M. truncatula* that is rapid and reproducible in order to test the hypotheses that cellular injury decreases as the acclimation temperature to which plants are exposed decreases. We evaluated two *M. truncatula* genotypes under four acclimation regimes to determine their maximum freezing tolerance under controlled conditions. Growth and development were measured concurrently to understand the physiology of cold acclimation in *M. truncatula*.

Materials and Methods

PLANT MATERIAL. Two *M. truncatula* genotypes, Jemalong-6, derived from the Australian **cultivar** Jemalong, and W6 5018, a wild accession from Italy, were used for this study. Seeds of Jemalong-6 were obtained from INRA (Institut National de la Recherche Agronomique, Mauguio, France) and W6 5018 from the USDA National Plant Germplasm System (NPGS), Western Plant Introduction Center, Pullman, WA. Jemalong-6 is one of the parents of a mapping population that has been analyzed for morphogenetic traits and QTL analysis in *M. truncatula* (Julier et al., 2007). Our previous study showed that W6 5018 had an erect plant type, was late flowering, and produced high biomass while Jemalong-6 was early flowering, produced decumbent shoots and low biomass under simulated autumn conditions (unpublished results). We used these two phenotypically contrasting genotypes to assess genotypic differences in cold acclimation. In alfalfa, cultivars with short, prostrate growth in autumn tend to be winter-hardy, while those with erect shoots and rapid growth are not (Sheaffer et al., 1992).

GROWTH CONDITIONS. Seeds were scarified using medium grain sandpaper one day before seeding into individual plastic pots (capacity of ≈2500 cm^3) filled with Sunshine Professional growing mix (SB300 Universal Mix, Sun Gro Horticulture Inc., Bellevue, WA, USA). The pots were placed in a greenhouse at 22 °C day/18 °C night with a 16-h photoperiod (Moreau et al., 2006). A total of 260 pots, 130 of each genotype, were seeded with five seeds per pot. Pots were thinned to one seedling after 2 weeks. After all plants developed at least five leaves (≈4 weeks), they were transferred to growth chambers and allocated to one of three temperature regimes.

Plants of each genotype were divided into three groups; 60 in non-acclimation (NA), 35 in cold acclimation regime 1 (CA1) and 35 in cold acclimation regime 2 (CA2). Genotypes were assigned to positions on growth chamber benches using a completely random experimental design. The temperatures, photoperiod, and photosynthetic photon flux (*PPF*) applied to each growth regime, together with the duration plants were exposed to those conditions, are presented in Table 1. After plants in the CA2 treatment were sampled for freezing tolerance, they were transferred to another chamber for cold acclimation 3 (CA3) regime, where they were further exposed to still lower temperature of 3.5 °C day/-1 °C night for 1 week at 180 *PPF* and 16-h photoperiod and sampled for freezing tolerance of CA3 regime. In CA3, plants were uniformly sprayed with ice-water mix to initiate ice-nucleation, 3 times in alternate days, during the treatment time. Growth chamber temperatures were monitored using a HOBO logger (Onset Inc. Pocasset, MA, U.S.). Light intensity was routinely monitored by a quantum sensor (LI-185, Li-COR Inc., Lincoln, NE, U.S.) throughout the experiment and the height of lights from the top of the plant canopy adjusted to maintain constant intensity. Plants in all chambers were fertilized with Peters Excel [15N-2.2P-12.5K (The Scotts Company, Marysville, OH, U.S.)] diluted 100-fold with distilled water. Each plant received ≈250 mL of nutrient solution every two weeks and was lightly watered every day.

DETERMINATION OF FREEZING TOLERANCE. Ion leakage was used to assess freezing tolerance (FT) and cold acclimation in *M. truncatula* by exposing tissue to a laboratory controlled freeze-thaw protocol. Ion leakage and injury assessment were patterned after Peng et al. (2007). Freezing tolerance of non-acclimated plants was measured three times (week 1, week 2, week 3) after the treatments began, in order to

determine developmental influences on the tolerance, if any. Freezing tolerance of plants in the CA regimes was measured at the end of the treatment period, after 4 weeks for CA1, 5 weeks for CA2, and 6 weeks for CA3. The freezing test temperatures were as follows: -1, -2, -3, -4, -5, -6, -7 °C for NA; -3, -5, -6, -7, -8, -9, -10, -11, -13, -15 °C for CA1; -5, -7, -9, -10, -11, -12, -13, -15, -17 °C for CA2; -4, -6, -8, 10, -12, -14, -16, -18, -20 °C for CA3. For each test temperature, two leaves of similar age (the first fully expanded leaf from the tip) were sampled from each of three replicate plants for a total of six replications and were placed in test tubes. Six unfrozen samples of each genotype were placed on ice, as unfrozen controls. Treatment samples were placed in a glycol bath at -1°C for 1 h, after which, freezing was initiated by ice-nucleation produced by dropping a small ice crystal into each tube and kept for 1 h before further lowering the temperature.

The temperature in glycol bath was lowered at 1 °C/40 min and the frozen samples were removed at each test temperature and thawed in ice overnight. On the following day, the samples were removed from ice and kept at 4 °C for 1.5 h and at 22 °C (room temperature) for another 1 h. Twenty mL of deionized water was added to each sample, which were then vacuum infiltrated four times (1 min each) and placed on a gyratory platform shaker (InnOva-2300, New Brunswick Sci. Co. Inc., Edison, NJ, U.S.) at 250 rpm for 1.5 h. An initial IL measurement was then taken with a 3100 conductivity meter (YSI Inc., Yellow Springs, OH, U.S.). Samples were autoclaved at 121 °C for 20 min with a slow exhaust cycle of 0.45 $kg \cdot min^{-1}$ and after being brought to room temperature, the total IL was measured. Initial leakage was expressed as percentage of the final IL value, and percent leakage for each treatment was converted to percentage injury as;

$$\% \text{ injury} = [\% \text{ IL (t)} - \% \text{ IL (c)}/100 - \% \text{ IL (c)}] \text{ X } 100$$

where, % IL (t) and % IL (c) are measurements of percentage of ion leakage from the respective freeze-treatment temperature and the unfrozen control, respectively. LT_{50}, a measure of FT, was derived for both the genotypes in all growth regimes by determining the freeze test temperature at which 50% injury (midpoint of maximum and minimum % injury) occurred (Fig. 1), as explained in Lim et al. (1998).

GROWTH EVALUATIONS. Number of leaves and length of the primary stem were measured on four randomly selected plants from each treatment (NA, CA1, and CA2) at weekly intervals for the first three weeks after treatments began. Plants were sampled for dry weights at FT sampling time of each growth regime. Roots, stems and leaves of each plant were separated and dried for 3 days at 60 °C to estimate the dry weight (Hoy et al., 2002). Leaf area (LA) was measured using a Li-3100 Area Meter (Li-COR, Inc., Lincoln, NE, U.S.). Specific leaf area (SLA, leaf area per leaf dry weight) and root-to-shoot ratio (RS) were also determined on each sample.

STATISTICAL ANALYSIS. The experimental design was a completely randomized design with genotypes and temperature treatments as fixed factors. Statistical analyses were conducted using the GLM procedure of SAS (version 8; SAS Institute, Cary, NC, USA). Differences between the treatment means were separated by Fisher's protected least significance (LSD) test at 0.05 and 0.01 probability levels. Regressions were performed separately for each genotype at different growth regimes using freeze test temperatures and PI data.

Results

DEVELOPMENT OF FREEZING TOLERANCE. The two *M. truncatula* genotypes maintained at NA and CA regimes varied for leaf PI with test temperatures (Fig. 2, A-D). For NA plants, no effect of sampling time (week 1, week 2, or week 3) or genotype x sampling time interaction was evident, so only one sampling time (week 2) was used to compare the results with CA regimes (Fig. 2A). Non-acclimated tissues did not suffer any injury when frozen at -1 °C (data not shown), had the lowest injury at -2 °C, and suffered incrementally more pronounced injury with decreasing test temperatures (Fig. 2A). Non-acclimated plants of the two genotypes differed in injury only at -3 °C when W6 5018 showed 10% injury compared to 23% for Jemalong-6. The maximum injury recorded for non-acclimated tissues was about 90% at -7 °C.

Leaves from CA1 plants did not show the incidence of freeze injury until -3 °C (Fig. 2B). However, freezing injury increased steadily from -5 to -10 °C, after which, there was no further increase in injury in either genotype. A similar trend was observed at CA2 (Fig. 2C). The temperatures at which genotypes differed varied among cold treatments, with CA1 [-5, -7 and -8 °C (Fig. 2B)] and with CA2 [-5, -9 and -10 °C (Fig. 2C)]. In contrast to non-acclimated samples, Jemalong-6 had higher freezing tolerance than W6 5018, as it suffered lower freeze injury at test temperatures in CA1 and CA2 at which the genotypes differed (Fig. 1 B and C). Interestingly, the differences in PI between the two genotypes were apparent at almost every test temperature in CA3 (Fig. 2D). In addition, the difference between genotypes in PI widened in CA3 with decreasing test temperatures, reaching 65% in W65018 but only 39% in Jemalong-6 at the lowest test

temperature (-20 °C). Although the injury increased steadily with decreasing test temperatures at CA3 regime, it did not reach a plateau for either genotype.

Freezing tolerance (LT_{50}) for both genotype and its interaction with growth regimes were not significant. Freezing tolerance of the both *M. truncatula* genotypes varied with growth regime (Table 2). Within the NA growth durations (week1, week2 and week3), FT of the genotypes was similar, with LT_{50} about -3.0 °C. However, LT_{50} temperatures of all the CA regimes were lower than the non-acclimated plants. Freezing tolerance of plants grown at CA1 and CA2 regimes did not differ, as evident by similar average $LT_{50,}$ -7.6 and -7.7 °C, respectively, across genotypes. When the CA2 plants were exposed to a lower temperature (3.5 °C day/-1 °C night) for an additional week (CA3 regime), the FT of the plants were increased by almost ≥ -4 °C. Whereas two genotypes exhibited difference in their cold acclimation ability (Fig. 2B-D), cultivar difference in LT_{50} was not evident in non-acclimated or in cold-acclimated leaf tissues. Consequently, LT_{50} values differentiated FT between non-acclimated and cold-acclimated plants but was not an effective measure of discriminating the tolerance between the two *M. truncatula* genotypes before or after cold acclimation.

GROWTH EVALUATIONS. Number of leaves per plant and stem length varied with growth regimes (Fig. 3, A and B). Stem length was reduced drastically in CA1 and CA2 compared to NA regimes at every week (Fig. 3A) during the first three weeks after treatment regimes began, but to a smaller extent between the CA1 and CA2 regimes. Similar results were also observed for number of leaves per stem (Fig. 3B). In general, W6 5018 produced longer stems than Jemalong-6 under NA and CA2 (week 2) while this

difference between the genotypes narrowed in CA1 regime. Jemalong-6 developed average of one leaf more than W6 5018 in CA1 while they had similar number of leaves in CA2 growth regime (Fig. 3B).

Dry weights of the two genotypes did not differ at the first week of NA regime although W6 5018 had higher leaf area compared to Jemalong-6 (Table 3). The difference in dry weights between the two genotypes was apparent at week 2 and week 3, and at both times, W6 5018 had higher leaf dry weight (LDW), stem dry weight (SDW) and root dry weight (RDW) compared to Jemalong-6. SLA and RS remained the same for both the genotypes at all NA growth durations. However, both the genotypes produced similar dry weights, LA, RS and SLA at CA1 and CA2 regimes. W6 5018 produced higher SDW and LA compared to Jemalong-6 at CA3 regime but both the genotypes had similar LDW, RDW and RS. Comparing the non-acclimated plants to cold-acclimated plants, it was clear that CA treatments reduced the growth of the plants as evident by reduced dry weights in both genotypes. Plants grown for 6 weeks in CA3 regime produced much lower dry weights than week 3 plants. CA3 plants had comparable LDW and SDW, but higher RDW and RS compared to week 2 NA plants. However, the dry weights of CA1 plants with only 4 weeks of growth were comparable to 3 weeks old NA plants. These results clearly indicate that temperature had larger effect in dry weights than the growth duration at any CA growth regime. However, both the genotypes had comparable LA but reduced SLA at each cold acclimation regime.

Discussion

Ion leakage test performed on leaf tissue after freeze-thaw event was efficient for determining both FT and acclimation ability differences in the two genotypes of *M. truncatula*. Cell membranes are critical sites of freezing injury in plants. Numerous factors are involved in membrane damage at freezing temperatures, such as, cellular dehydration (lamellar-to-hexagonal–II phase transitions), expansion-induced lysis, and damage caused by reactive oxygen species, protein denaturation, etc. (Thomashow, 1999). Thus, protection of the cell membrane damage from such factors is important to improving FT. Cold acclimation induces metabolic changes in tissues to maintain membrane fluidity and to prevent membrane against freeze-dehydration (Graham and Patterson, 1982; Leborgne et al., 1992; Perras and Sarhan, 1989)

There was a substantial reduction in membrane injury in cold-acclimated plants compared to non-acclimated, indicating that CA regimes were effective. In previous literature (Antolín et al., 2005; Hekneby et al., 2006), 10 °C day/5 °C night temperature regime was used to assess CA and frost tolerance in *M. truncatula*. Acclimation temperatures used in our studies were comparable to those studies (CA1), but in addition, we included lower temperatures (7 °C day/4 °C night) in CA2 regime. However, both PI and FT (LT_{50}) measured in CA1 and CA2 plants were similar. This result suggests that the extended growth at lower temperature did not contribute to the hardiness level of the plants. Sakai and Larcher (1987) modeled the seasonal CA process in woody plants from temperate zones in two stages. The first stage proceeds at 10 to 20 °C in fall, involves accumulation of organic substances and attributes to a relatively small (-10 °C) level of

hardiness in plants. Similarly, CA1 and CA2 plants had increased FT in our studies within the range of temperatures.

Our results indicate that *M. truncatula* has a considerable ability to cold acclimate at subzero temperature. Plants acclimated in CA3 regime (3.5 °C day/-1 °C night) for one week, exhibited lowest injury compared to CA1 and CA2 at any test temperature. The significantly lower % injury observed in CA3 plants indicates that the subzero temperature exposure during CA3 was largely effective in inducing higher CA in the plants. In addition, we demonstrated a significant decrease of LT_{50} temperature in both genotypes, from about -3 °C in non-acclimated to ≥ -13 °C in cold-acclimated (CA3) plants. Freezing at subzero temperature is the second phase hardening (sub-zero hardening) wherein cold-acclimated plants enhance FT further than that achieved during hardening with above freezing temperatures. Second phase hardening has been documented in alfalfa (Castonguay et al., 1993), as well as other species, including wheat *(Tritium aestivum* L.) (Herman et al, 2006), rye (Oline, 1984) (*Secale cereale* L.), and in barley and oat *(Hordeum vulgare* L. and *Avena sativa* L.) (Livingston, 1996). Plants withstand dehydration and the physical effects of ice formation in the intercellular spaces during the second phase of CA by changing cell constituents and structures (Saxe et al., 2001; Weiser 1970). Distinct changes in translatable mRNA in alfalfa cultivars acclimated at subzero temperatures are associated with achieving maximal hardiness during snow cover in winter (Castonguay et al., 1993).

Ability to acclimate at subzero temperature is important for winter survival and spring growth for *M. truncatula*. In the present study, plants in CA2 developed a smaller, but significant FT while those in CA3 (subzero temperature) acquired still greater FT. These

results imply that the events in induction of CA in *M. truncatula* may also be explained by the biphasic process, as in woody plants and indicate its potential for winter survival. By contrast, Brandsæter et al. (2000) reported poor frost resistance and survival in *M. truncatula* acclimated for 2 weeks at 2 °C. However in their study, plants had already reached the flowering stage before they were frozen and this could be the reason for poor survival. The most effective temperature in inducing CA depends on species, tissues and developmental stages of the plant. A temperature slightly above 0 °C and slight below zero (-3 °C) are the two essential steps for developing full frost hardiness (Sakai and Larcher, 1987). Our CA3 regime included both the levels of temperature and the plants developed the two distinct levels of CA capabilities. However, neither genotype reached a plateau for % injury in CA3 regime. Further studies using the treatment temperatures colder than -20 °C in the freeze test may provide the maximum cold acclimation ability in this species under artificially controlled CA regimes.

In comparative analyses, freezing injury in W6 5018 initiated and reached the maximum earlier than Jemalong-6 in all CA regimes. Webb et al. (1994) demonstrated genotypic differences in leaf injury, rate of increase in freezing tolerance and achievement of maximal LT_{50} in spring oat, winter oat and winter rye acclimated at 2 °C for 4 weeks which is consistent with our results. Although the freezing tolerance of the two genotypes did not vary in cold acclimation regimes used in the study, the percent injury in Jemalong-6 was significantly lower than W6 5018 at most of the test temperatures in CA3 (Table 2). In addition, the initiation and maximum freeze injury recorded in Jemalong-6 were at lower freeze test temperature compared to W6 5018. These results clearly indicate that Jemalong-6 had superior cold acclimation ability than

W6 5810 under the CA regimes used in the study. Castonguay et al. (1993) showed differential cold-inducible gene products in contrasting winter-hardy alfalfa cultivars and suggested a close association between gene expression and FT within cultivars. Genotypic differences in CA abilities observed in this study implies that there may be differences in genes or expression of genes involved in regulatory and sensing mechanisms in controlling cold acclimation process in *M. truncatula*.

Exposure to different growth regimes resulted in differential growth responses in *M. truncatula*. Reduced number of leaves and stem length were observed in cold-acclimated plants within a week of initiation of CA regimes (Fig. 3). Low temperature reduces photosynthetic carbon fixation and sucrose synthesis which reduces growth and phloem export of sucrose in plants (Strand et al., 1999). At low temperatures, reduction in photosynthesis is considered as a major cause for growth cessation as indicated by reduced chlorophyll content and photosynthetic capacities in sensitive maize genotypes (Haldimann, 1998; Leipner et al., 1999). However, overwintering cereals can grow and survive at low temperatures and have the capacity to increase or maintain high photosynthesis for increased FT (Hurry et al., 1994). They use most of the photosynthates for energy and the carbon skeleton needed for metabolic accumulation for both cryoprotection and storage as adaptive responses. The observed reduced growth and development during the first three weeks in cold-acclimated plants in the present study, therefore, may indicate the allocation of more photosynthates for the induction of adaptive processes compromising the normal growth. Genotypes differed clearly in terms of growth and phenological development at NA while they had similar performance at CA regimes (Fig. 3 and Table 3). However, Jemalong-6 suffered lower reduction in

growth and development of Jemalong-6 was reduced less in CA regimes compared to its growth in NA regime than did W6 5018. These results further suggest that Jemalong-6 may have a relatively lower temperature requirement to attain maximum acclimation and thereby explain its significantly greater cold acclimation ability at CA3 regimes.

We observed reduction in leaf dry weight from CA1 to CA3 while increase in RDW, resulting in a higher RS ratio in both genotypes. Higher partitioning of dry matter in roots than in shoots has been reported in many species under low temperature stress (Wilson, 1988). Low shoot growth maybe due to low absorption and transportation of water and nutrients by roots at low temperature (BassiriRad et al., 1991). Higher RS ratio may also reveal a greater partitioning of carbon to the nutrient-absorbing tissues in low temperature conditions (Chapin, 1974). High SLA measured in non-acclimated plants in this study may indicate that the plants may have high rate of CO_2 exchange per unit leaf area and thus may have achieved the higher growth. High SLA has been regarded as an important trait in crops, such as sugarcane (Terauchi and Matsuoka, 2000) and winter cereal (Richards, 2000) attributing to high light interception during early growth and development. However, increase in leaf density (lower SLA) has been demonstrated as common response to low temperature in both chilling sensitive (*Phaseolus vulgaris* L., *Zea mays* L.) and chilling tolerant species (*Pisum sativum* L., *Spinacia oleracea* L.), which is consistent with our results (Wolfe, 1991). Reduced LA and SLA observed in CA regimes maybe adaptive strategies by which the cold-acclimated plants minimize leaf injury, reducing the leaf expansion, thereby reducing the surface area exposed to low temperature.

In conclusion, the present study clearly demonstrated that *M. truncatula* has an ability to cold acclimate as evident by significant increase in FT with the exposure to specific CA regimes (accompanied by reduced growth). Quantifying FT by measuring ion leakage in leaves proved to be a successful strategy as the difference in acclimation conditions and genotypes were well demonstrated with this approach. Since the two genotypes significantly differed in their freezing injury at various subzero test temperatures, while still exhibiting similar LT_{50} at CA regimes, we chose to present both the measurements in this study. Notably this result implies that LT_{50} may not be a comprehensive parameter to differentiate CA abilities among *M. truncatula* genotypes and that actual freezing injury at given test temperatures should be used in addition to LT_{50} to evaluate this trait in *M. truncatula*. Various CA regimes (CA1, CA2, and CA3) had differential effect on inducing cold hardening. Overall, the maximum FT was achieved under CA3 treatment, the only regime that included subzero temperature exposure. Thus, CA3 appears to be the best regime of the three regimes tested, to induce maximal CA under controlled conditions in *M. truncatula* for physiological and biochemical studies.

Literature Cited

Antolín, M.C., M. Hekneby, and M. Sánchez-Díaz. 2005. Contrasting responses of photosynthesis at low temperatures in different annual legume species. Photosynthetica 43:65-74.

Arora, R. and J. Palta. 1991. A loss in the plasma membrane ATPase activity and its recovery coincides with incipient freeze-thaw injury and postthaw recovery in onion bulb scale tissue. Plant Physiol. 95:846-852.

Arora, R. and M.E. Wisniewski. 1994. Cold acclimation in genetically related (Sibling) deciduous and evergreen peach [*Prunus persica* (L.) Batsch]: II. A 60-Kilodalton bark protein in cold-acclimated tissues of peach is heat stable and related to the dehydrin family of proteins. Plant Physiol. 105:95-101.

Arora, R., L.J. Rowland, and G.R. Panta. 1997. Chill-responsive dehydrins in blueberry: Are they associated with cold hardiness or dormancy transitions? Physiologia Plantarum 101:8-16.

BassiriRad, H., J.W. Radin, and K. Matsuda. 1991. Temperature-dependent water and ion transport properties of barley and sorghum roots: I. Relationship to leaf growth. Plant Physiol. 97:426-432.

Brandsæter, L.O., T. Smeby, A.M. Tronsmo, and J. Netland. 2000. Winter annual legumes for use as cover crops in row crops in Northern regions: II. Frost resistance study. Crop Sci. 40:175-181.

Brummer, E.C. 2004. Applying genomics to alfalfa breeding programs. Crop Sci. 44:1904-1907.

Castonguay, Y., P. Nadeau, and S. Laberge. 1993. Freezing tolerance and alteration of translatable mRNAs in alfalfa (*Medicago sativa* L.) hardened at subzero temperatures. Plant Cell Physiol. 34:31-38.

Castonguay, Y., S. Laberge, E. C. Brummer, and J. J. Volenec. 2006. Alfalfa winter hardiness: A research retrospective and integrated perspective. Adv. in Agron. 90:203-265.

Chapin, F.S., III. 1974. Morphological and physiological mechanisms of temperature compensation in phosphate absorption along a latitudinal gradient. Ecology 55:1180-1198.

Graham, D. and B. Patterson. 1982. Responses of plants to low, nonfreezing temperatures: proteins, metabolism, and acclimation. Annual Review of Plant Physiology 33:347-372.

Haagenson, D.M., S.M. Cunningham, and J.J. Volenec. 2003. Root physiology of less fall dormant, winter hardy alfalfa selections. Crop Sci. 43:1441-1447.

Haldimann, P. 1998. Low growth temperature-induced changes to pigment composition and photosynthesis in *Zea mays* genotypes differing in chilling sensitivity. Plant, Cell & Environment 21:200-208.

Hekneby, M., M.C. Antolín, and M. Sánchez-Díaz. 2006. Frost resistance and biochemical changes during cold acclimation in different annual legumes. Environmental and Experimental Botany 55:305-314.

Herman, E.M., K. Rotter, R. Premakumar, G. Elwinger, R. Bae, L. Ehler-King, S. Chen, and D.P. Livingston, III. 2006. Additional freeze hardiness in wheat acquired by exposure to -3°C is associated with extensive physiological, morphological, and molecular changes. J. Exp. Bot. 57:3601-3618.

Hoy, M.D., K.J. Moore, J.R. George, and E.C. Brummer. 2002. Alfalfa yield and quality as influenced by establishment method. Agron J. 94:65-71.

Hurry, V.M., G. Malmberg, P. Gardestrom, and G. Oquist. 1994. Effects of a short-term shift to low temperature and of long-term cold hardening on photosynthesis and

ribulose-1,5-bisphosphate carboxylase/oxygenase and sucrose phosphate synthase activity in leaves of winter rye (*Secale cereale* L.). Plant Physiol. 106: 983-990.

Julier, B., T. Huguet, F. Chardon, R. Ayadi, J.-B. Pierre, J.-M. Prosperi, P. Barre, and C. Huyghe. 2007. Identification of quantitative trait loci influencing aerial morphogenesis in the model legume *Medicago truncatula*. TAG Theoretical and Applied Genetics 114:1391-1406.

Koster, K.L. and D.V. Lynch. 1992. Solute accumulation and compartmentation during the cold acclimation of Puma rye. Plant Physiol. 98:108-113.

Leborgne, N., L. Dupou-Cezanne, C. Teulieres, H. Canut, J.-F. Tocanne, and A.M. Boudet. 1992. Lateral and rotational mobilities of lipids in specific cellular membranes of *Eucalyptus gunnii* cultivars exhibiting different freezing tolerance. Plant Physiol. 100:246-254.

Leipner, J., Y. Fracheboud, and P. Stamp. 1999. Effect of growing season on the photosynthetic apparatus and leaf antioxidative defenses in two maize genotypes of different chilling tolerance. Environ. Exper. Bot. 42:129-139.

Lim C.C., R. Arora, and E. C. Townsend (1998). Comparing Gompertz and Richrads functions to estimate freezing injury in jury in *Rhododendron* using electrolyte leakage. J Amer. Soc.Hort. Sci. 123:246-252.

Livingston, D.P., III. 1996. The second phase of cold hardening: Freezing tolerance and fructan isomer changes in winter cereal crowns. Crop Sci. 36:1568-1573.

Marian, C.O., S.L. Krebs, and R. Arora. 2004. Dehydrin variability among rhododendron species: a 25-kDa dehydrin is conserved and associated with cold acclimation across diverse species. New Phytologist 161:773-780.

McKenzie, J. S., R. Paquin, and S. H. Duke. 1988. Cold and heat tolerance, p.259-30. In A. A. Hanson, D. K. Barnes, and R. R. Hill, Jr. (eds.) Alfalfa and alfalfa improvement, Agron. Monogr. 29. ASA-CSSA, and SSSA, Madison, WI.

Moreau, D., C. Salon, and N. Munier-Jolain. 2006. Using a standard framework for the phenotypic analysis of *Medicago truncatula*: an effective method for characterizing the plant material used for functional genomics approaches. Plant, Cell & Environment 29:1087-1098.

Nunes, M.E.S. and G.R. Smith. 2003. Electrolyte leakage assay capable of quantifying freezing resistance in rose clover. Crop Sci. 43:1349-1357.

Oline, C.R. 1984. An adaptive response of rye to freezing. Crop Sci. 24:51-54.

Palta, J., B. Whitaker, and L. Weiss. 1993. Plasma membrane lipids associated with genetic variability in freezing tolerance and cold acclimation of *Solanum* species. Plant Physiol. 103:793-803.

Patton, A.J., S.M. Cunningham, J.J. Volenec, and Z.J. Reicher. 2007. Differences in freeze tolerance of Zoysiagrasses: II. Carbohydrate and proline accumulation. Crop Sci. 47:2170-2181.

Peng, Y., W. Lin, W. Cai, and R. Arora. 2007. Overexpression of a *Panax ginseng* tonoplast aquaporin alters salt tolerance, drought tolerance and cold acclimation ability in transgenic Arabidopsis plants. Planta 226:729-740.

Perras, M. and F. Sarhan. 1989. Synthesis of freezing tolerance proteins in leaves, crown, and roots during cold acclimation of wheat Plant Physiol. 89:577-585.

Richards, R.A. 2000. Selectable traits to increase crop photosynthesis and yield of grain crops. J. Exp. Bot. 51:447-458.

Sakai, A. and W. Larcher. 1987. Frost survival of Plants: Responses and adaptation to freezing stress. Springer-Verlag Berlin Heidelberg. Ecological Studies.62.

Saxe, H., M. Cannell, O. Johnsen, M. Ryan, and G. Vourlitis. 2001. Tree and forest functioning in response to global warming. New Phytologist 149:369-399.

Sheaffer, C.C., D.K. Barnes, D.D. Warnes, W.E. Lueschen, H.J. Ford, and D.R. Swanson. 1992. Seeding-year cutting affects winter survival and its association with fall growth score in alfalfa. Crop Sci. 32:225-231.

Strand, A., V. Hurry, S. Henkes, N. Huner, P. Gustafsson, P. Gardestrom, and M. Stitt. 1999. Acclimation of arabidopsis leaves developing at low temperatures. Increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiol. 119:1387-1398.

Sulc, R., K. Albrecht, and S. Duke. 1991. Leakage of intracellular substances as an indicator of freezing injury in alfalfa. Crop Sci. 31:430-435.

Terauchi T. and Matsuoka M. 2000. Ideal characteristics for the early growth of sugarcane. Jpn. J. Crop Sci. 69:286-292.

Teutonico, R., J. Palta, and T. Osborn. 1993. In vitro freezing tolerance in relation to winter survival of rapeseed cultivars. Crop Sci. 33:103-107

Thomashow, M. 1999. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol. 50:571-599.

Wanner, L.A. and O. Junttila. 1999. Cold-induced freezing tolerance in *Arabidopsis*. Plant Physiol. 120:391-400.

Webb, M.S., M. Uemura, and P.L. Steponkus. 1994. A comparison of freezing injury in oat and rye: two cereals at the extremes of freezing tolerance. Plant Physiol. 104:467-478.

Weiser, C.J. 1970. Cold resistance and injury in woody plants: knowledge of hardy plant adaptations to freezing stress may help us to reduce winter damage. Science 169:1269-1278.

Welling, A., T. Moritz, E.T. Palva, and O. Junttila. 2002. Independent activation of cold acclimation by low temperature and short photoperiod in hybrid aspen. Plant Physiol. 129:1633-1641.

Wilson, J.B. 1988. A Review of evidence on the control of shoot: root ratio, in relation to models. Ann Bot. 61:433-449.

Wolfe, D.W. 1991. Low temperature effects on early vegetative growth, leaf gas exchange and water potential of chilling-sensitive and chilling-tolerant Crop Species. Ann Bot. 67:205-212.

Zhu, Y., C. C. Sheaffer, and D. K. Barnes. 1996. Forage yield and quality of six annual *Medicago* species in the North-Central USA. Agron. J. 88:955-960.

Table 1. Air temperature (°C day/night), photoperiod (h), photosyntetic photon flux (*PPF*), and growth duration (weeks) of *M. truncatula* genotypes, Jemalong-6 and W6 5018, at non acclimation (NA; 3 weeks) and cold acclimation (CA1-4 weeks; CA2-5 weeks; CA3-6 weeks) growth regimes. CA2 plants were transferred to CA3 regime for a week at lower temperature (3.5 °C day/-1 °C night).

Growth regimes	Temperature [°C (day/night)]	Photoperiod (hour)	PPF ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Duration (week)
Non-acclimation (NA)	22/18	16	400	3
Cold-acclimation 1 (CA1)	18/10	16	400	2
	10/5	16	400	2
Cold acclimation 2 (CA2)	18/10	16	400	1
	10/5	16	400	2
	7/4	16	180	2
Cold acclimation 3 (CA3)	18/10	16	400	1
	10/5	16	400	2
	7/4	16	180	2
	3.5/-1	16	180	1

Table 2. Freezing tolerance (LT_{50}) of *M. truncatula* genotypes, W6 5018 and Jemalong-6, as influenced by non-acclimation [NA (22 °C day/18 °C night)] at a weekly interval (week 1, week 2, week 3) and cold acclimation (CA1, CA2, CA3) regimes. Measurements in CA1 (18 °C day/10 °C night for 2 weeks followed by 10 °C day/5 °C night for 2 weeks), CA2 (18 °C day/10 °C night for 1 week followed by 10 °C day/5 °C night for 2 weeks and 7 °C day/4 °C night for 2 weeks) and CA3 (CA2 regime followed by 3.5 °C day/-1 °C night for 1 week) were taken after 4, 5 and 6 weeks, respectively, after the treatments. Mean LT_{50} values ±SD are shown for 3 replicate plants. LT_{50} [°C (mean ±SD)] was derived as [(max% injury – min% injury)/ 2] + min% injury.

	$(LT_{50})^z$	
Growth regime	W65018	Jemalong-6
NA		
Week 1	-3.4 ±0.49a	-3.1 ±0.36a
Week 2	-2.9 ±0.23a	-2.9 ±0.40a
Week 3	-3.4 ±0.15a	-3.6 ±0.10a
CA		
CA1	-7.3 ±0.53b	-8.1 ±0.43b
CA2	-7.5 ±0.61b	-7.9 ±0.25b
CA3	≥-13.3 ±2.43c	≥-12.3 ±2.1c

[z] LT_{50} is expressed as temperature at which 50% injury occurred.

LT_{50} ± SD with different letters are significantly different based on LSD values (P = 0.01).

Table 3. Leaf area (LA), specific leaf area (SLA), root-to-shoot ratio (RS), leaf dry weight (LDW), stem dry weight (SDW) and root dry weight (RDW) of *M. truncatula* genotypes, W6 5018 and Jemalong-6**,** at non-acclimation (NA; week 1, week2, week 3) and cold acclimation (CA1, CA2, CA3) regimes.

Regime	Genotype	LA [z] (cm^2)	SLA [z] ($cm^2.g^{-1}$)	RS [z] (ratio)	LDW [z] (g/plant)	SDW [z] (g/plant)	RDW [z] (g/plant)
NA							
Week1	W6 5018	108a	178a	1.08a	0.61a	0.42a	1.19a
	Jemalong-6	73b	173a	1.20a	0.42a	0.33a	0.89a
Week2	W6 5018	194a	154a	0.94a	1.28a	0.99a	2.19a
	Jemalong-6	163a	186a	0.89a	0.91b	0.79a	1.48b
Week3	W6 5018	281a	105a	0.82a	2.7a	3.31a	4.91a
	Jemalong-6	194b	128a	0.89a	1.5b	1.51b	2.72b
CA							
CA1	W6 5018	116a	74a	0.63a	1.67a	1.27a	1.98a
	Jemalong-6	139a	83a	0.68a	1.68a	1.54a	2.16a
CA2	W6 5018	82a	83a	0.98a	1.01a	0.95a	1.99a
	Jemalong-6	73a	88a	1.39a	0.83a	0.83a	2.31a
CA3	W6 5018	108a	108a	1.32a	1.00a	1.13a	3.04a
	Jemalong-6	93b	122a	1.84a	0.76a	0.77b	2.70a
LSD(0.05) among regime means		24.5	35.4	0.31	0.27	0.33	1.09

[z] Mean values of genotype (n = 4) for response at a particular regime. Values within a regime with different letters are significantly different based on LSD ($P < 0.05$).

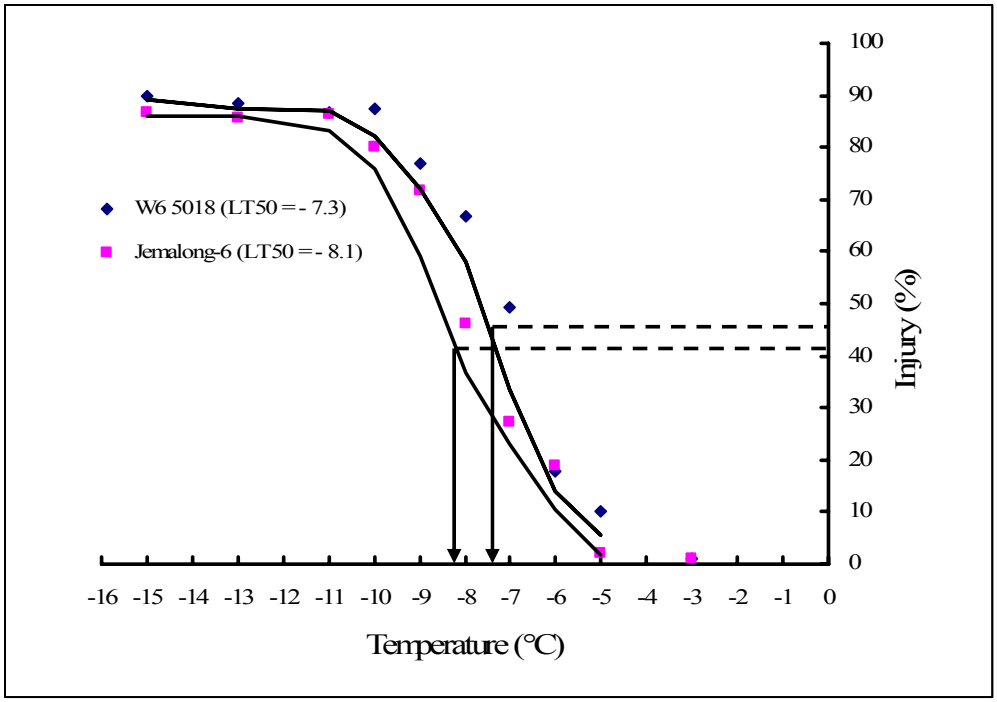


Figure 1. Freezing tolerance (LT_{50}) of *M. truncatula* genotypes, W6 5018 and Jemalong-6 at cold acclimation (CA1) growth regime. The dashed lines show 50% injury of a particular genotype and the bold arrows show the respective LT_{50} temperature. LT_{50} was derived as [(max% injury – min% injury)/ 2] + min% injury. Injury was measured at each test temperature in leaves samples after 4 weeks of CA1 growth regime.

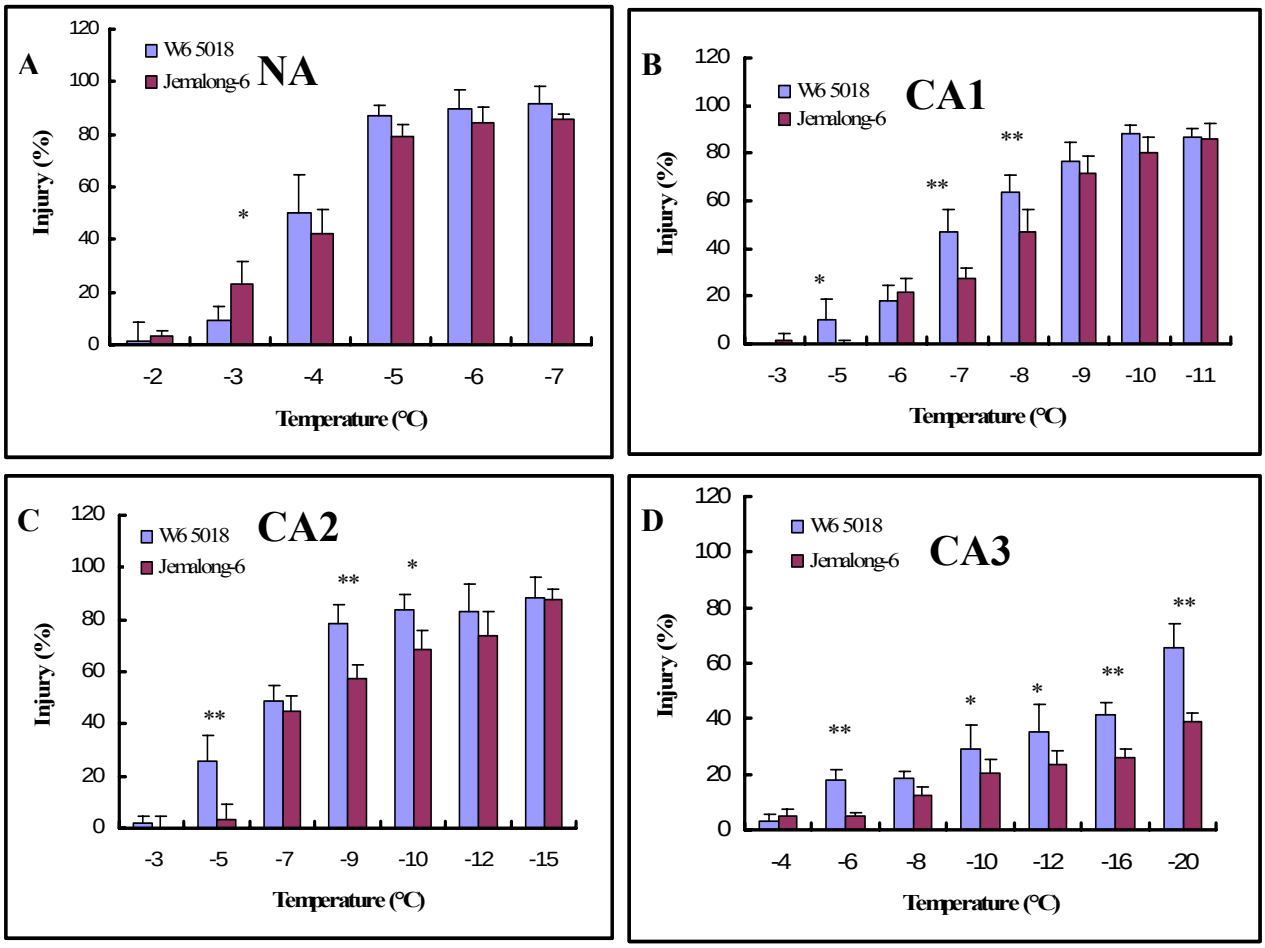


Figure 2. Percent injury measured at different freeze test temperature in leaves of *M. truncatula* genotypes, W6 5018 and Jemalong-6, at non acclimation, NA (A), cold acclimation 1, CA1 (B), cold acclimation 2, CA2 (C), and cold acclimation 3, CA3 (D) regimes. Measurements in CA1 (18 °C day/10 °C night for 2 weeks followed by 10 °C day/5 °C night for 2 weeks), CA2 (18 °C day/10 °C night for 1 week followed by 10 °C day/5 °C night for 2 weeks and 7 °C day/4 °C night for 2 weeks) and CA3 (CA2 regime followed by 3.5 °C day/-1 °C night for 1 week) were taken after 4, 5 and 6 weeks, respectively, after the treatments. Measurement in NA (22 °C day/18 °C night) was taken at the first week under the treatment. The vertical bars represent ±SD of 6 replications. Significant differences between the two genotypes at 0.05 and 0.001 confidence levels at a particular test temperature are marked with * and **, respectively.

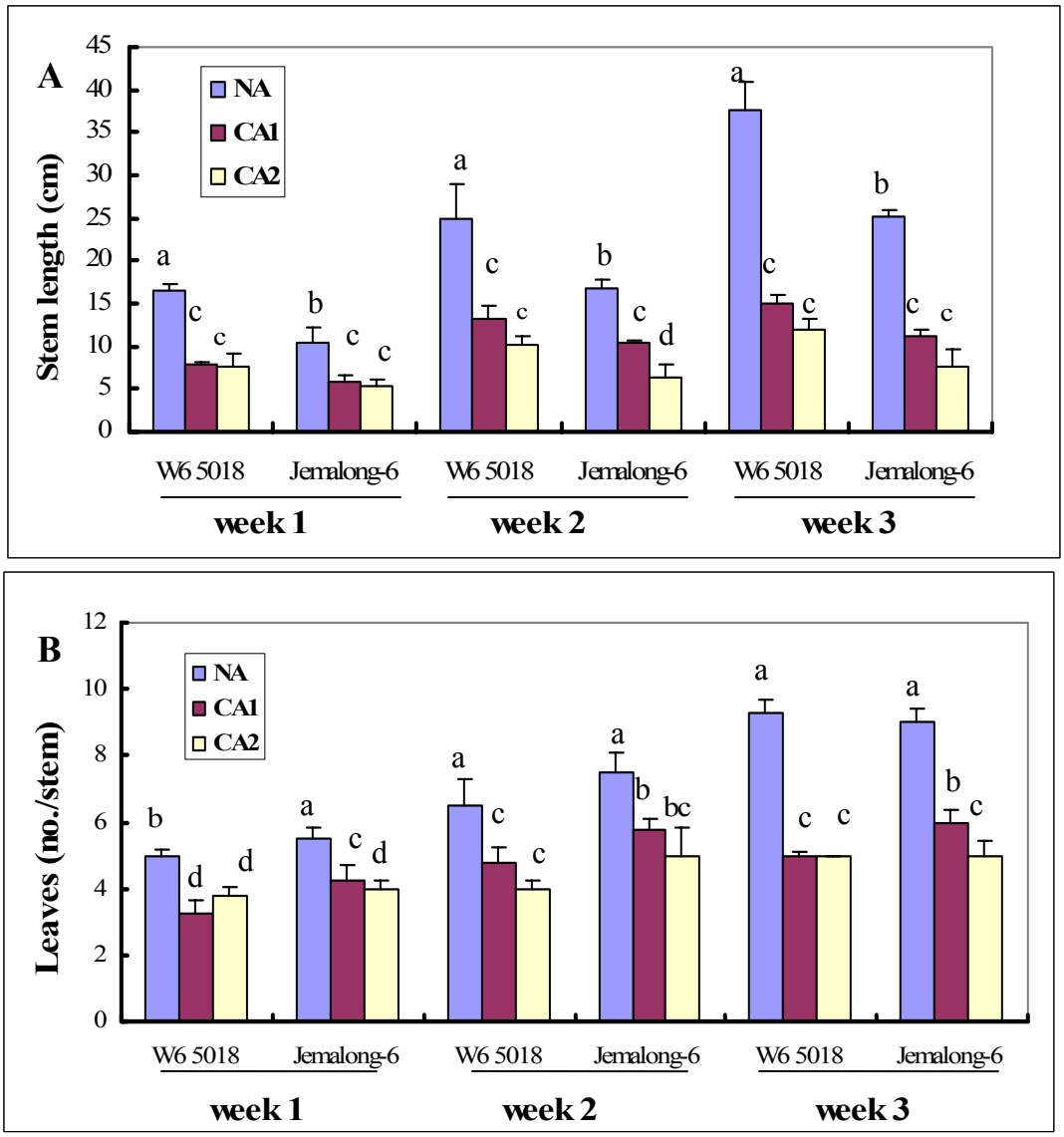


Figure 3. Effect of growth regimes (non-acclimation, NA; cold acclimation1, CA1, and cold acclimation, CA2) on stem length (A) and number of leaves on the first primary stem (B) of *M. truncatula* genotypes, W65018 and Jemalong-6, during the first three weeks (week 1, week 2, and week 3). Means with the same letters in each week were not significantly different based on LSD ($P < 0.05$). The vertical bars represent ±SD based on mean of 4 replications.

CHAPTER 4. COLD ACCLIMATION ALTERS SOLUBLE SUGAR CONCENTRATION AND COMPOSITION IN *MEDICAGO TRUNCATULA*

An article to be submitted to Crop Science

Babita Thapa, Allen D. Knapp, Rajeev Arora and E. Charles Brummer

ABSTRACT

Medicago truncatula Gaertn., an annual relative of alfalfa, could be used to determine genetic basis of cold acclimation in alfalfa and other legume crops, but the physiological processes associated with cold acclimation and freezing tolerance are not well understood in this species. We studied differences in sugar concentrations and composition in leaf, stem and root tissues of *M. truncatula* in non-acclimation (NA) and after cold-acclimation (CA) treatments to begin to assess their role in freezing tolerance (FT) and as a potential screening tool for FT. Two genotypes, Jemalong-6 and W6 5018, were grown in three non-acclimation (NA1, NA2, NA3) and cold acclimation (CA1, CA2, CA3) regimes. Among the soluble sugars analyzed, fructose, glucose, sucrose, and total sugar (sum of all soluble sugars in a sample) exhibited distinct increases following CA treatments, with the maximum concentration following the CA3. Raffinose was only present in CA regimes while stachyose was not detected in any treatment samples. Trehalose concentration did not vary between NA and CA regimes. The highest total sugars concentrations were found in the stems. Jemalong-6 had higher stem raffinose, leaf trehalose, sucrose and total sugar concentrations than W6 5018 in CA3 regime. A strong correlation between leaf soluble sugars and leaf FT suggested that sugars may have a role in CA or predict the development of CA. Leaf total sugar concentration was positively correlated with percent water content in the leaf after cold acclimation. The

presence of raffinose may be indicative of FT as it was only present in CA regimes and not detected in NA regimes.

INTRODUCTION

Understanding cold acclimation (CA) is important for concurrently increasing autumn yield and winter survival in alfalfa [(*Medicago sativa* L.); Brummer et al., 2000]. Such investigations are difficult in alfalfa because of its complex genetic makeup, and outcrossing with inbreeding depression. *Medicago truncatula* Gaertn is an annual self-pollinated legume and is a model plant for the study of legume biology (Cook, 1999). *M. truncatula*, a relative of alfalfa with a small genome size and a high level of biodiversity (Thoquet et al., 2002), could be used to dissect the genetics of CA if its ability for CA is characterized. Furthermore, *M. truncatula* is exposed to cool weather during the growing season in its native mediterranean region (Sultan et al., 2001) and therefore it is plausible that it has evolved mechanisms to tolerate low temperatures.

Cold acclimation lowers the temperature at which the plants are damaged by freezing temperatures through physiological and metabolic adjustments and improves freezing tolerance (Guy, 1990). Freezing injury occurs as a result of destabilized membranes from freeze-induced dehydration (Steponkus, 1984) and thus, maintaining membrane stability is a key function of CA (Thomashow, 1999). Acclimation to cold involves synthesis of proteins, lipids, enzymes and sugars metabolites that confer protection from cold (Graham and Patterson, 1982). Sugars maintain the liquid crystalline state of the membrane bilayer, stabilize proteins under freeze-induced dehydration, and reduce membrane injury (Uemura and Steponkus, 2003). Increases in cellular sugar concentrations also lower the temperature at which the cell freezes, and decreases the

damage from freeze-induced osmotic dehydration (Levitt, 1980). Both of those functions improve the overall FT in the plants.

Changes in carbohydrate levels and types coinciding with changes in freezing tolerance have led to the conclusion that alterations in carbohydrates are important components of plants ability to survive low winter temperatures (Castonguay et al., 1995; Dionne et al., 2001; Chen et al., 1980). Sucrose is the most commonly accumulated sugar in response to low temperature (Guy, et al., 1992). In Zoysiagrass, rhizomes and stolons (*Zoysia spp.*) higher concentration of total reducing sugars and glucose are associated with increased FT measured during cold acclimation using 4 wk of 8/2°C day/night treatment. Sucrose concentrations in crown tissues in annual bluegrass (*Poa annua L.*) increased at temperatures below freezing and maximum concentration coincided with maximum FT (Dionne et al., 2001). Increased FT in buffalograss [Buchloe dactyloides(Nutt.) Engelm.] stolons was correlated with higher fructose, glucose and sucrose levels (Ball et al., 2002).

Castillo et al. (1990) have proposed that raffinose family oligosaccharides (RFOs), which include raffinose, stachyose and verbascose, play roles in CA. Like sucrose, RFOs are important storage and phloem transport carbohydrates for many plants (Sprenger and Keller, 2000). In alfalfa roots, the concentration of RFO and galactinol synthase transcripts, increased in October and November in winter hardy field grown plants (Cunnigham et al., 2003). Koster and Lynch (1993) proposed that sucrose and raffinose accumulated external to vacuole in puma rye leaves (Secale cereale L. cv Puma) during CA may have cryoprotective effects on the plasma membrane.

Biosynthesis and function of trehalose as an energy reserve, transport sugar and stress protectant are discussed in a recent paper (Wingler, 2002), though it was previously found to exert a toxic effect in *Cruscuta reflexa* Roxb. vines because it interfered the synthesis of cell wall polysaccharides in shoot tips (Veluthambi et al., 1982) Transgenic rice plants that expressed trehalose-6-phosphate synthase and trehalose-6-phosphate synthase phosphates showed higher ability to tolerate trehalose synthesis and resulted in increased tolerance to drought, salt and cold stress as shown by chlorophyll fluorescence and growth inhibition analyses (Jang et al., 2003). Building stress tolerance through overproduction of trehalose has been considered as a novel strategy for improving crop plants (Penna, 2003).

Increase in total soluble sugar (TSS) in roots, stems, and leaves was demonstrated in *M. truncatula* after cold-acclimation at 10/5°C day/night for 4-6 wk using colorimetric method of sugar determination (Sanchez-Diaz et al., 2000; Hekneby et al., 2004). However, the relative concentrations of each sugar and whether they change with different CA induction regimes is not known. In addition, it is unclear whether such changes in sugars preceding CA may relate to any measureable alteration of FT in *M. truncatula*. We determined accumulation of soluble sugars, more specifically, concentrations of trehalose, glucose, fructose, sucrose, raffinose and stachyose in roots, stems and leaves in *M. truncatula* under different cold acclimation regimes. Relationship between sugar concentrations at acclimation regimes and freezing tolerance (LT_{50}; temperature at which 50% injury occurred) was characterized.

MATERIALS AND METHODS

Plant material

Two *M. truncatula* genotypes, Jemalong-6, derived from the Australian cultivar Jemalong, and W6 5018, a wild accession from Italy, were used for this study. Seeds of Jemalong-6 were obtained from INRA (Institut National de la Recherche Agronomique, Mauguio, France) and W6 5018 from the USDA National Plant Germplasm System (NPGS), Western Plant Introduction Center, Pullman, WA. More information on characteristics of the accessions is found in Thapa et al. (2008).

Growth conditions

Seeds were scarified using medium grain sandpaper one day before seeding into individual plastic pots (capacity of ≈2500 cm^3) filled with Sunshine Professional growing mix (SB300 Universal Mix, Sun Gro Horticulture Inc., Bellevue, WA, USA). The pots were placed in a greenhouse at 22 °C day/18 °C night with a 16-h photoperiod (Moreau et al., 2006). A total of 260 pots, 130 of each genotype, were seeded with five seeds per pot. Pots were thinned to one seedling after 2 weeks. After all plants developed five leaves (≈4 weeks), they were transferred to growth chambers and randomly assigned to one of three temperature regimes.

Plants of each genotype were divided into three groups; 60 in non-acclimation (NA), 35 in cold acclimation regime 1 (CA1) and 35 in cold acclimation regime 2 (CA2). Genotypes were randomly assigned to positions on growth chamber benches. The temperatures, photoperiod, and photosynthetic photon flux (*PPF*) applied to each growth regime, together with the duration plants were exposed to those conditions, are presented

in Table 1. After plants in the CA2 treatment were sampled for freezing tolerance, they were transferred to another chamber for cold acclimation 3 (CA3) regime, where they were further exposed to still lower temperature of 3.5 °C day/-1 °C night for 1 week at 180 units *PPF* and 16-h photoperiod and sampled for freezing tolerance of CA3 regime.

Sugar extraction and analysis

Soluble sugars including trehalose, glucose, fructose, raffinose and stachyose were extracted from 25 mg of ground lyophilized leaf, stem and root materials. The sum of all the soluble sugars in each sample is reported as total sugar in the study. Sugars were extracted using ethanol extraction patterned after Zhu at al. (2006). A DX500 Dionex high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) system was used. This system consisted of a GP-50 gradient pump and ED40 electrochemical detector (Dionex Corporation, Sunnyvale, CA). Sugars were separated on a Dionex CarboPac PA-10 analytical column (4 X 250 mm) followed by an amino trap (4 X 50 mm), and eluted isocratically at a flow rate of mobile phase (120 mM NaOH) of 1 mL min^{-1} for 25 min. The electrode potential sequence for the detector was; E_1= 0.05 V; cleaning potential E_2= 0.60 V; electrode regeneration potential E_3= -0.60 V. Samples were injected via an AS50 automated sampler with a 25-µL sample loop. The mobile phase, 120 mM NaOH solution was prepared by diluting carbonate–free 50% (w/w) NaOH solution in deionized distilled water which was previously degassed under vacuum with a water bath sonicator (FS140, Fisher Scientific, Pittsburgh, PA) for 30 min. Sugar standards (Sigma) were used to identify the sugars based on their retention times and for standard curve development.

Determination of Freezing Tolerance

Details of ion leakage (IL) and injury assessment procedures are explained in Thapa et al.(2008). Ion leakage was used to assess freezing tolerance (FT) and cold acclimation in *M. truncatula* by exposing tissue to a laboratory controlled freeze-thaw protocol. LT_{50}, a measure of FT, was derived as explained in Lim et al. (1998) using IL data.

Growth Evaluations

Four randomly selected plants were used for biomass measurement at FT sampling time of each growth regime. Roots, stems and leaves of each plant were separated and fresh weights of the tissues were measured before they were dried in oven (60°C for 72 hrs) for dry weight measurements. Leaf percent water content (PWC) was determined leaf fresh (LFW) weight and leaf dry weight (LDW) on a fresh weight basis [(LFW-LDW)/LFW X 100]. Leaf area (LA) was measured using a Li-3100 Area Meter (Li-COR, Inc., Lincoln, NE, U.S.). Specific leaf area (SLA, leaf area per leaf dry weight) and root-to-shoot ratio (RS) were also determined on each sample. The Minolta model SPAD 502 (Minolta, Ramsey, NJ) chlorophyll meter was used to estimate chlorophyll content (Chl) on the three fully expanded leaves at the apex and the average value was used for data analysis.

Statistical analysis

The experimental design was a completely randomized design with genotypes, tissue types and temperature treatments as fixed factors. Statistical analyses were conducted using the GLM procedure of SAS (version 8; SAS Institute, Cary, NC, USA).

Differences between the treatment means were separated by Fisher's protected least significance (LSD) test at 0.05 and 0.01 probability levels. Pearson correlation coefficients (*r*) were calculated by PROC CORR.

RESULTS

Soluble Sugar concentrations and composition in various plant tissues

Concentrations of sugars varied considerably ($P < 0.01$) among the different plant tissues (Table 2). Stem tissues had highest sucrose, glucose and total sugars concentrations compared to leaf and root tissues. However, trehalose concentrations in stems were lower than in roots. Fructose concentration in stem and leaf were similar but were higher than in root tissues. Root had the lowest glucose, fructose and sucrose concentrations. Raffinose concentrations did not vary between leaf and stem tissues. Total soluble sugars varied distinctly among the tissues with highest concentration determined in stem followed by leaf and root tissues. Among all the soluble sugars, sucrose and trehalose were present in highest and lowest concentrations, respectively, in all tissues types.

Leaf, stem and root tissues varied in soluble sugars composition (Table 2). Only stems tissues were composed of all the soluble sugars evaluated in this study. Trehalose was undetected in leaf tissues and raffinose was undetected in root and also in NA stem and leaf tissues.

Soluble sugar concentrations at CA and NA growth regimes

Leaf glucose increased considerably among CA regimes (Fig. 1A). CA regimes induced differential glucose concentrations but NA regimes did not alter the concentration. In CA3, the concentration of glucose reached 25 mg g^{-1} dry dwt. which was almost 5-fold

higher than in NA regimes (~5 mg g^{-1} dry wt.). Similar results were observed in leaf fructose concentrations (Fig. 1B). Sucrose concentrations also increased in all CA regimes compared to NA regimes (Fig. 1C). The concentrations were similar in CA1 and CA2 regimes but increased in CA3 regime. Although average leaf raffinose concentrations were the highest in CA1, the concentration was similar to CA3 regime (Fig. 1D). The total sugars in leaf tissues increased distinctly at three CA regimes with pronounced (3-fold) increase in CA3 compared to NA regimes.

Stem soluble sugar concentrations were similar among the NA growth regimes while they clearly differed in CA regimes (Fig. 2A-F). Concentration of stem glucose, fructose and total sugars were highest in CA3 and they vary distinctly at each CA regime (Fig. 2A-B and E). Sucrose and trehalose concentrations were highest in CA3 compared to CA1 and CA2 regimes but the concentrations were similar in CA1 and CA2 regimes (Fig. 2 C and E). Likewise, raffinose concentrations in stem varied considerably among the CA regimes, ranging from average of 0.10 mg-1 g dwt in CA1 to 0.22 mg-1 g dwt in CA3 (Fig. 2D).

Root glucose, fructose, sucrose and total sugars were highest in CA3 among all the growth regimes (Fig.3A-E). Concentrations of glucose and fructose varied between CA1 and CA2 while sucrose and total sugars remained the same. In NA regimes, sucrose and total sugars were higher in NA1 compared to NA2 and NA3. Root trehalose concentrations did not vary among the CA regimes but were comparable to NA1 and NA3 regimes (Fig. 3D).

Differences in soluble sugar concentrations and composition in genotypes

The two genotypes Jemalong-6 and W6 5018 did not differ in leaf glucose, fructose and total sugars concentrations in NA regimes, except for sucrose concentration in NA1 (Fig. 1A-E). In contrast, genotypic differences in leaf sugar concentrations were apparent in all CA regimes, except for glucose in CA3. Jemalong-6 had substantially higher fructose, sucrose and total sugars compared to W6 5018 in both CA1 and CA3 regimes while the opposite was true in CA2 regime (Fig. 1A-E). Jemalong-6 also attained higher raffinose concentration than W6 5018 in CA1 regime (Fig. 1D).

Jemalong-6 produced higher stem fructose, glucose and total sugars in CA1 regimes and sucrose in CA3 regimes than W6 5018 (Fig. 2A-D). Stem raffinose and trehalose concentrations were also higher in Jemalong-6 compared to W6 5018 in CA3 regime while their concentrations were comparable in all the other regimes (Fig. 2D and E).

Roots of Jemalong-6 accumulated higher glucose, sucrose and total sugars than W6 5018 at CA3 regime (Fig. 3A, C and E) whereas W6 5018 attained higher fructose concentration than Jemalong-6 at CA1 and CA2 regimes (Fig.3B). The highest trehalose concentration was detected in W6 5018 at NA2 in root tissues.

Relation of soluble sugars to freezing tolerance and other growth parameters in *M. truncatula*

Growth traits, IN, Chl, LDW, SDW and RDW were negatively correlated with total sugars in NA with coefficients ranging from -0.42 to -0.50 (Table 3). However, LT_{50}, RS and PWC were not correlated with the total sugar concentrations in non-acclimated leaf

tissues. Glucose and fructose were strongly related to reduction in IN and LDW as indicated by high negative correlation coefficients while the traits were not affected by leaf sucrose concentrations. Growth traits, such as, IN, SDW and RDW were not correlated with total sugar concentrations in CA but LDW was negatively correlated (-0.49). Correlations of between each sugar, glucose, fructose, sucrose including the total sugars in leaf tissues were significant with Chl, SLA, RS and PWC in CA plants. The correlation between the freezing tolerance (LT_{50}) and the total sugars in the CA leaf tissues was negative but the strongest (r = -0.94) among all the parameters evaluated. sugars.

DISCUSSION

Cold acclimation induced accumulation of soluble sugars in *M. truncatula*. Variation in sugars concentrations among tissues, with the highest concentration in stem observed in this study are consistent with the results from Sánchez-Díaz et al.(2000). Sugar accumulation has been one of the most prominent metabolic changes observed during CA in many plant species (Cunningham et al., 2003; Hekneby et al., 2006). Cold acclimation initiates with perception of non-freezing low temperature following the temporal sequences, starting with membrane rigidification and cytoskeleton rearrangements and ending with product formation, such as certain proteins, amino acids, sugars etc., that confer freezing tolerance on the plant (Bjorn and Dhindsa, 2002). In addition, sugar accumulation may occur through starch hydrolysis as activity of starch- degradative enzymes, α–amylase and β-amylase, are enhanced at low temperatures, 15 and 10 °C,

respectively (Kacperska-Palacz, 1978). These evidences indicate that plants preferentially synthesis sugar during low temperature conditions.

Comparisons of leaf sugars accumulations at the different CA regimes reveal that each sugar and the total sugars have reached their maximal levels at the CA3 regime while the CA1 and CA2 had similar sugar concentrations (Fig 1). Interestingly, CA3 regime had significantly increased freezing tolerance of genotypes compared to CA1 and CA2 in our previous study . These results may imply sugar concentrations at low nonzero and subzero temperatures may play important roles inducing the two levels of FT in *M. truncatula*. Previously, the existence of two stages of hardening has been reported in field-grown winter wheat (Yoshida et al., 1997), winter rape (Kacperska, 1993) and in woody perennial (Sakai and Larcher, 1987). In the first stage of CA in woody plants, synthesis of starch reserve occurs for substrate and energy required for metabolic changes at the second stage. Protein and membrane lipids are synthesized during the second stage leading to maximal hardiness. In herbaceous plants, sugars accumulations are important to acquire higher resistance as they do not accumulate starch during autumn (Sakai and Larcher, 1987). In winter wheat, the first stage of CA is associated with a reduction of weakly bound water due to increase in cellular substances but the second stage happens with increase in weakly or moderately bound water content (Yoshida et al., 1997). In the same context, significant increase in total sugars observed in CA3 may involve in osmotic regulation to avoid excessive desiccation, binding water molecules to decrease free water and increase in bound water to promote growth in the second stage of hardening. In addition, sugars may be required as substrate and for modulation of other

assimilate-requiring processes, such as COR gene expression, fatty acid desaturation and proline synthesis, during CA (Stitt and Hurry, 2002).

Sucrose contributed almost 52% of the total sugar in cold-acclimated leaf. Leaves of cold-hardy herbaceous winter rye and winter oilseed rape showed remarkable recovery of photosynthetic capacity with increased activitiy of Calvin-cycle enzymes but cold-sensitive species did not (Hurry et al. 1994, 1995). Increased expression and activity of sucrose synthesis enzymes correlated with an accumulation of sugars in leaves in those species. Glucose and fructose levels also increased following the trend of sucrose during CA in our study as has been reported in rice (Morsy et al, 2007) and winter rape (Hurry et al., 1995). This result may imply that sucrose may be cleaved into fructose and glucose with the greater invertase activity at low temperatures (Tromnsmo et al., 1993). Unlike Morsy et al. (2007), higher glucose and fructose concentrations were correlated with increased FT in our study. These low molecular weight sugars exhibit freezing point depression and protect membranes during CA (Santarius, 1982) and are essential to decrease cell water potential and to maintain turgor pressure (Strum, 1999).

Raffinose was detected in cold-acclimated leaves and stems but not in non-acclimated tissues in our study. To our knowledge there have been no reports about raffinose concentrations in *M. truncatula*. In frost-hardy *Ajuga reptans,* raffinose serves as a transport and a storage carbohydrate that can be induced to accumulate in the leaf mesophyll by cold treatment (Bachmann et al., 1994). The evidence for a role of raffinose in CA was demonstrated in *Arabidopisis thaliana* where its accumulation in leaf discriminated the more tolerant accession (Koltke et al., 2004). In alfalfa, differences in the maximum level of FT between non-hardy and winterhardy cultivars are related to the

capacities of plants to accumulate and synthesize raffinose in crowns at low temperature (Castonguay et al., 1995). The increased in raffinose was associated with marked increase in activity of galactinol synthase, a key enzyme in its metabolism (Castonguay and Nadeau, 1998). In our study, genotypic difference in raffinose concentrations was only apparent in stem tissues with the highest concentration at CA3 regime in both genotypes. It appears that raffinose synthesized in the leaf tissues get transported and stored in stems, as reserve which probably gets hydrolyzed producing sucrose as has been observed in some of the raffinose sink tissues (Madore, 1995)

We observed low concentrations of trehalose in stem and root tissues as has been reported in most vascular plants (Wingler, 2002), including rice (Garg et al., 2002). Despite its proposed role in protecting proteins and membranes during dehydration (Thevelien , 1996 , Dreennan et al., 1993), trehalose may not serve as a protectant against cold stress because its concentrations did not increase during CA in this study. Recent studies suggest the role of trehalose in coordinating carbon supply with plant growth and development (Ramon and Rolland, 2007). The trehalose-6-phosphate synthase gene (TPS 1) that synthesizes the intermediate of trehalose-6-phosphate (T6P) in trehalose pathway, is not only required for embryo development (Gomez et al., 2006) but also for normal vegetative development and floral transition (Van Dijken et al., 2004). High concentration of trehalose observed in our study may further support the role of trehalose in growth and development rather than as stress protectant in *M. truncatula.*

A strong correlation between total sugar and PWC in the CA leaf (Table 3) indicates that sugar accumulation helps in water relations by osmotic adjustment. In cold-grown winter rape leaves, increased frost tolerance subjected to subfreezing temperature,

is related to the decreased water potential of the tissue caused by changes in osmotic potential of the cells (Kacperska and Kulesza, 1987). Significant positive correlation between Chl and total sugars may indicate role of sugars in the enhancement of in photosynthetic carbon metabolism in CA. Further, greater leaf total sugar was associated with improved FT in this study (Table 3) may suggest that it is one of the several mechanisms of tolerance in *M. truncatula*. There are ample observations suggesting that accumulation of sugars has roles in enzyme cryoprotection, membrane stability and osmotic regulation during dehydration during freezing in cold-treated tissues (Xin and Browse, 2000; Koster and Lynch, 1992; Carpenter et al., 1986; Lineberger and Steponkus, 1980; Steponkus, 1984).

Based on our data, we conclude that CA with low positive temperature and subzero temperature formation of two distinct levels of sugars that discriminate the levels of FT in *M. truncatula*. Sugars may contribute cryoprotective function as the highest FT coincided with the highest concentration of sugars. In addition, increased sugars may aid in osmotic regulation and maintenance of normal metabolism as indicated by its positive correlation with PWC and chlorophyll content, SLA. Among the sugars, raffinose was only present in cold treated tissues and therefore its presence may have some importance contributions in improving the freezing tolerance in *M. truncatula* and the accumulation of raffinose, particularly in the stem, may be one of the important traits for freezing tolerance in *M. truncatula*. However, considering CA is a complex process, it is plausible that sugar composition and concentration may not solely contribute to FT through their known roles as osmotica and cryoprotection but their interactions with other processes of CA may also be involved for FT in *M. truncatula*.

Table 1. Air temperature (°C day/night), photoperiod (h), photosynthetic photon flux (*PPF*), and growth duration (wk) of *M. truncatula* genotypes, Jemalong-6 and W6 5018, at non acclimation (NA; 3 weeks) and cold acclimation (CA1-4 wk; CA2-5 wk; CA3-6 wk) growth regimes. CA2 plants were transferred to CA3 regime for a week at lower temperature (3.5 /-1°C day/night).

Growth regimes	Temperature [°C (day/night)]	Photoperiod (hour)	PPF ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Duration (week)
Non-acclimation (NA)	22/18	16	400	3
Cold-acclimation 1 (CA1)	18/10	16	400	2
	10/5	16	400	2
Cold acclimation 2 (CA2)	18/10	16	400	1
	10/5	16	400	2
	7/4	16	180	2
Cold acclimation 3 (CA3)	18/10	16	400	1
	10/5	16	400	2
	7/4	16	180	2
	3.5/-1	16	180	1

Table 2. Sugar composition and concentrations in leaf, stem and root tissues of *M. truncatula*. Values are the mean (n=48) across genotypes and growth regimes. nd, Not detected.

	Trehalose	**Glucose**	**Fructose**	**Sucrose**	**Raffinose**	**Total sugars**
			mg g^{-1} dry wt.			
Leaf	nd	11.3 b[Z]	10.2 a	27.5 b	0.153 a	49.16 b
Stem	0.033 b	21.6 a	10.5 a	30.07 a	0.156 a	62.21 a
Root	0.048a	2.4 c	1.2 b	28.9 b	nd	32.73c

[Z]Within columns, means followed by the same letter are not significantly different according to Fisher's protected LSD (α =0.05).

Table 3. Pearson correlations for growth traits [number of internodes (#IN), leaf dry weight (LDW, g plant^{-1}), shoot dry weight (SDW, g plant^{-1}), root dry weight (RDW, g plant^{-1}), root-to-shoot ratio (RS)], chlorophyll content (Chl), percent water content (PWC, %), and freezing tolerance (LT_{50})] with leaf soluble sugar concentrations of *M. truncatula* grown in nonacclimation (NA) and cold-acclimation (CA) regimes.

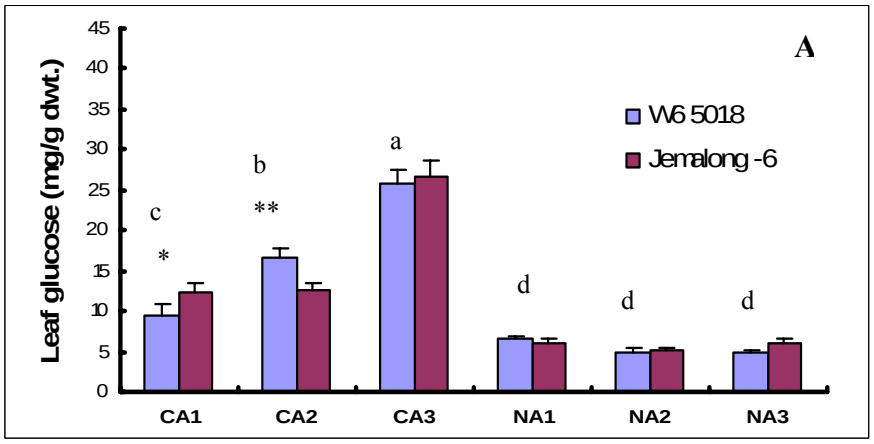
	IN	**Chl**	**LDW**	**SDW**	**RDW**	**SLA**	**RS**	**PWC**	**LT_{50}**
NA									
Glucose	-0.71***	-0.24ns	-0.64**	-0.59**	-0.51*	0.30ns	0.26ns	0.59**	0.02ns
Fructose	-0.71***	-0.31ns	-0.65***	-0.57**	-0.48*	0.33ns	0.35ns	0.63**	0.03ns
Sucrose	-0.12ns	-0.50*	-0.27ns	-0.30ns	-0.34ns	0.05ns	-0.09ns	0.04ns	0.09ns
Total Sugar	-0.42*	-0.44*	-0.50*	-0.46*	-0.47*	0.11ns	0.21ns	0.31ns	-0.15ns
CA									
Glucose	-0.02ns	0.66***	-0.52**	-0.36ns	0.37ns	0.73***	0.71***	0.63**	-0.82***
Fructose	0.00ns	0.60**	-0.46*	-0.31ns	0.38ns	0.73***	0.69***	0.64***	-0.90***
Sucrose	-0.20ns	0.59**	-0.48*	-0.34ns	0.39*	0.71***	0.69***	0.65***	-0.64**
Total sugar	-0.21ns	0.63***	-0.49*	-0.29ns	0.38ns	0.88***	0.70***	0.91***	-0.94***

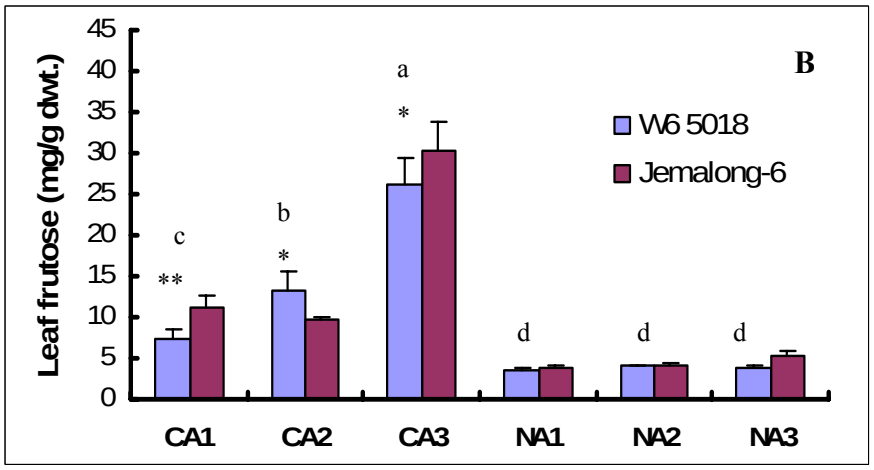
*Significant at $P < 0.05$

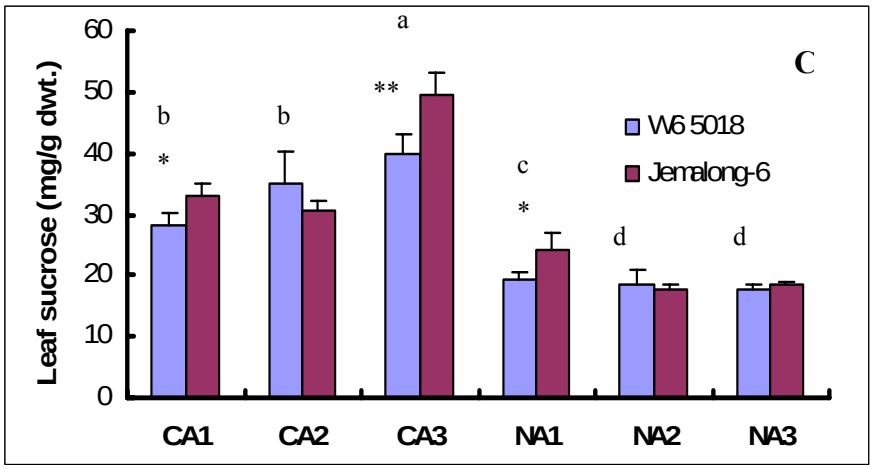
** Significant at $P < 0.01$

*** Significant at $P < 0.001$

ns, not significant


A
Leaf glucose (mg/g dwt.)
45
40
35
30
25
20
15
10
5
0
W6 5018
Jemalong -6
c
*
b
**
a
d
d
d
CA1
CA2
CA3
NA1
NA2
NA3


B
Leaf frutose (mg/g dwt.)
45
40
35
30
25
20
15
10
5
0
W6 5018
Jemalong-6
c
**
b
*
a
*
d
d
d
CA1
CA2
CA3
NA1
NA2
NA3


C
Leaf sucrose (mg/g dwt.)
60
50
40
30
20
10
0
W6 5018
Jemalong-6
b
*
b
a
**
c
*
d
d
CA1
CA2
CA3
NA1
NA2
NA3

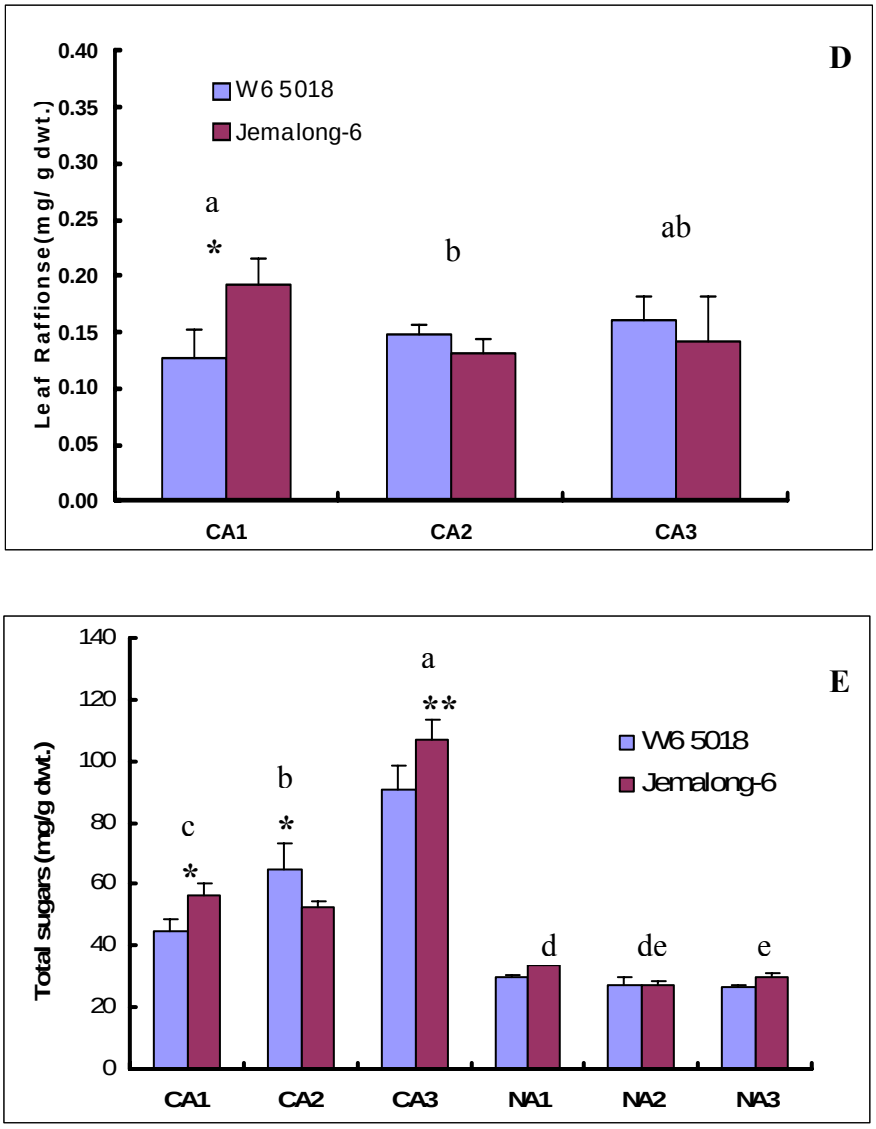
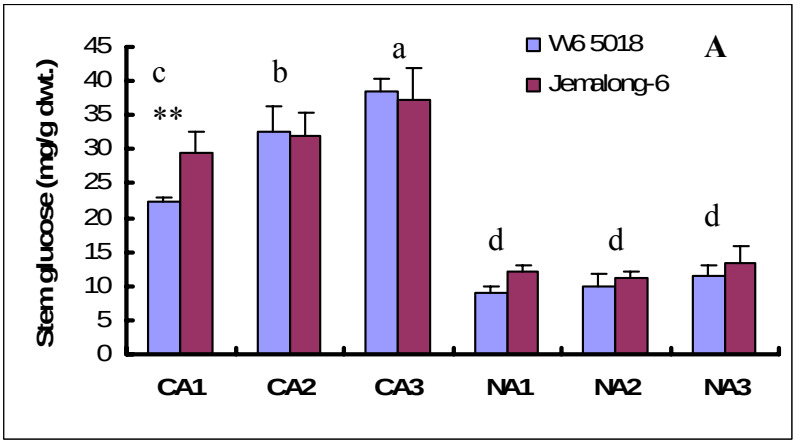
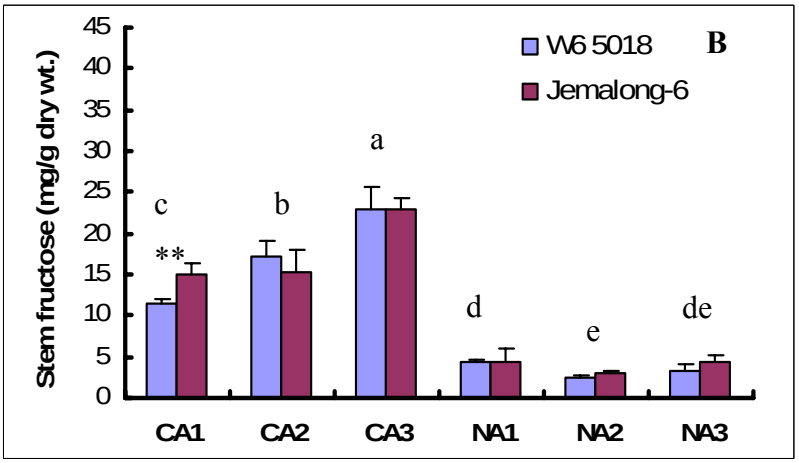
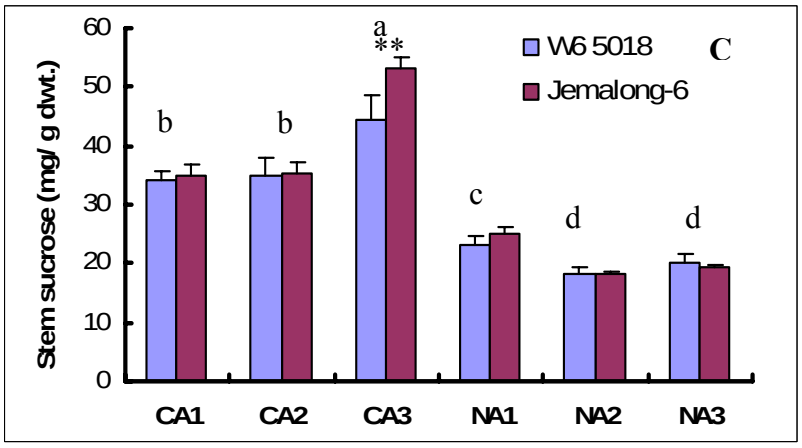


Figure 1. Concentrations of leaf glucose (A), fructose (B), sucrose (C), raffinose (D) and total sugars (E) in *M. truncatula* genotypes W6 5018 and Jemalong-6 at non-acclimation (NA1, NA2, NA3) and cold-acclimation (CA1, CA2, CA3) regimes. Vertical bars represent standard error of the mean (n=4). Growth regimes with the same letter are not significantly different according to Fisher's protected LSD (α =0.05). Asterisks indicate significant differences between the genotypes at P <0.01 (*) and <0.001 (**) within a growth regime.


A
W6 5018
Jemalong-6
Stem glucose (mg/g dwt.)
45
40
35
30
25
20
15
10
5
0
c
**
b
a
d
d
d
CA1
CA2
CA3
NA1
NA2
NA3


B
W6 5018
Jemalong-6
Stem fructose (mg/g dry wt.)
45
40
35
30
25
20
15
10
5
0
c
**
b
a
d
e
de
CA1
CA2
CA3
NA1
NA2
NA3


C
W6 5018
Jemalong-6
Stem sucrose (mg/ g dwt.)
60
50
40
30
20
10
0
b
b
a
**
c
d
d
CA1
CA2
CA3
NA1
NA2
NA3

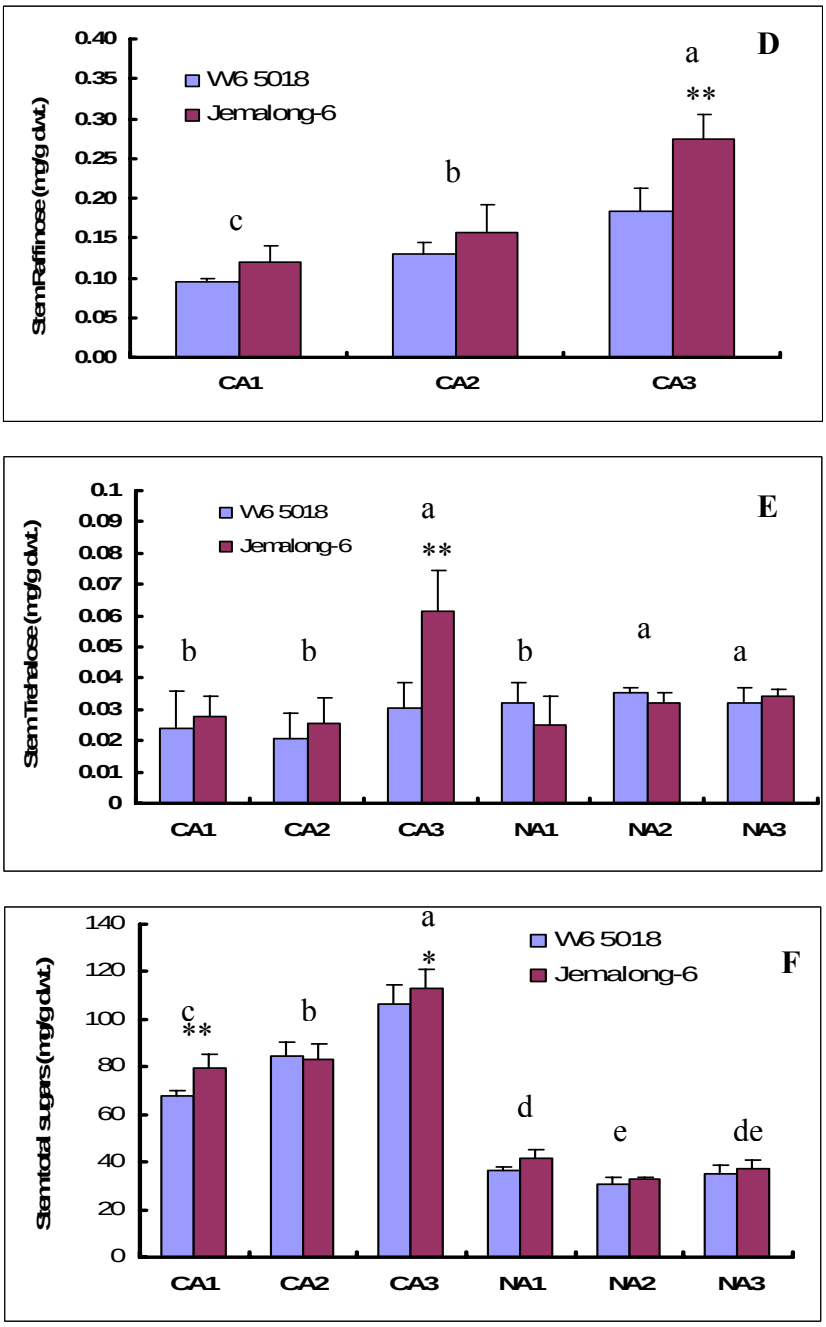
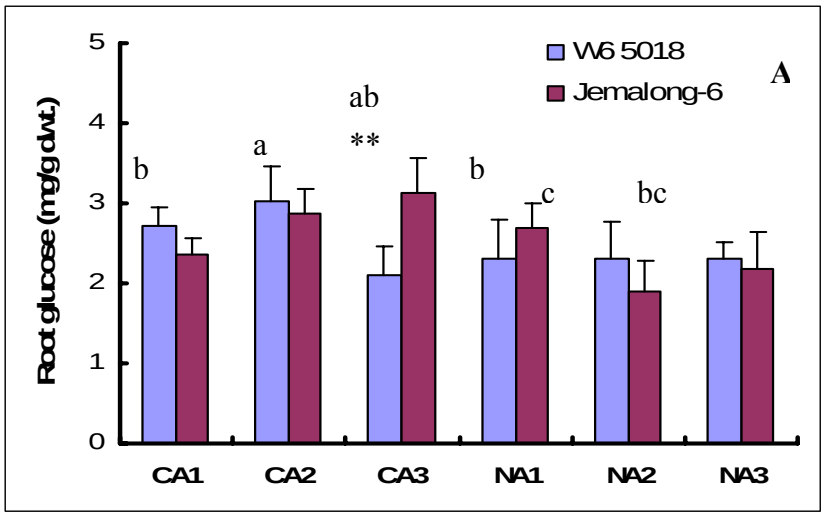
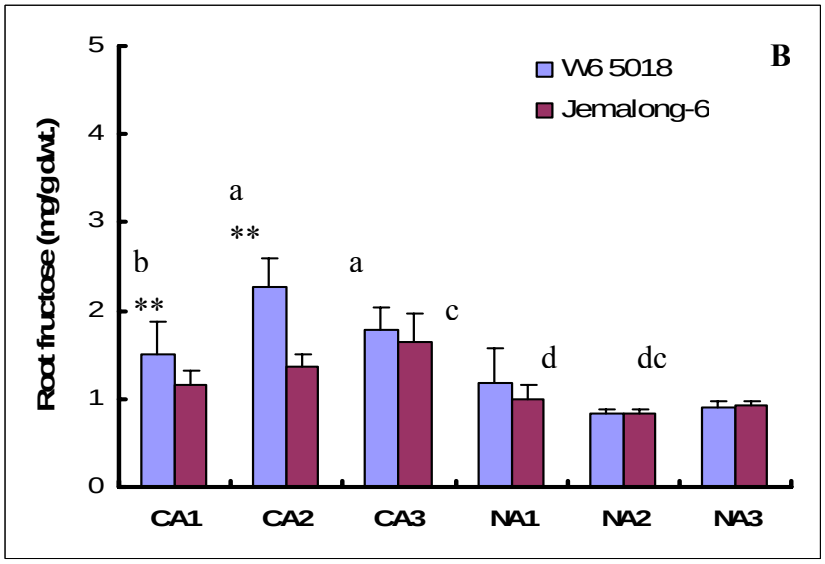
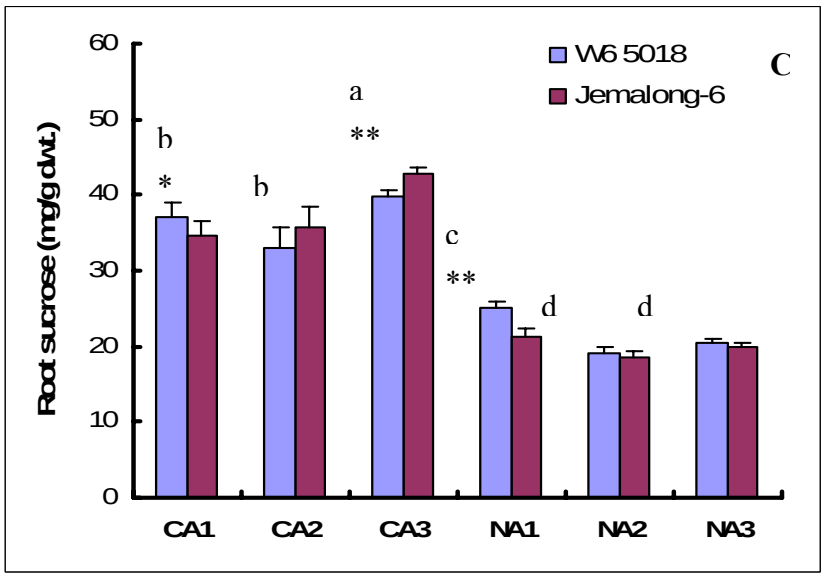
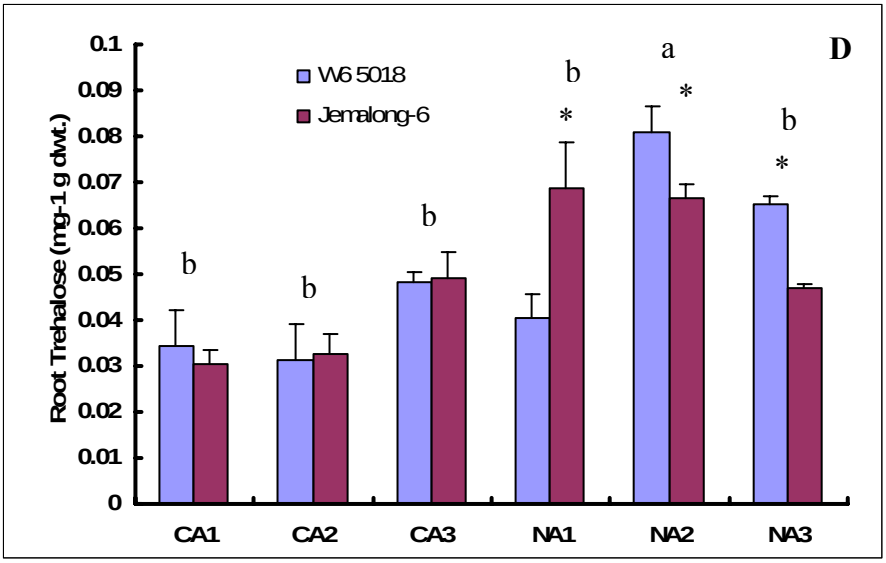


Figure 2. Concentrations of stem glucose (A), fructose (B), sucrose (C), raffinose (D), trehalose (E) and total sugars (F) stem tissues in *M. truncatula* genotypes W6 5018 and Jemalong-6 at non-acclimation (NA1, NA2, NA3) and cold-acclimation (CA1, CA2, CA3) regimes. Vertical bars represent standard error of the mean (n=4). Growth regimes with the same letter are not significantly different according to Fisher's protected LSD (α =0.05). Asterisks indicate significant differences between the genotypes at P <0.01 (*) and <0.001 (**) within a growth regime.


W6 5018
Jemalong-6
A
Root glucose (mg/g dwt.)
5
4
3
2
1
0
b
a
ab
**
b
c
bc
CA1
CA2
CA3
NA1
NA2
NA3


W6 5018
Jemalong-6
B
Root fructose (mg/g dwt.)
5
4
3
2
1
0
b
**
a
**
a
c
d
dc
CA1
CA2
CA3
NA1
NA2
NA3


W6 5018
Jemalong-6
C
Root sucrose (mg/g dwt.)
60
50
40
30
20
10
0
b
*
b
a
**
c
**
d
d
CA1
CA2
CA3
NA1
NA2
NA3



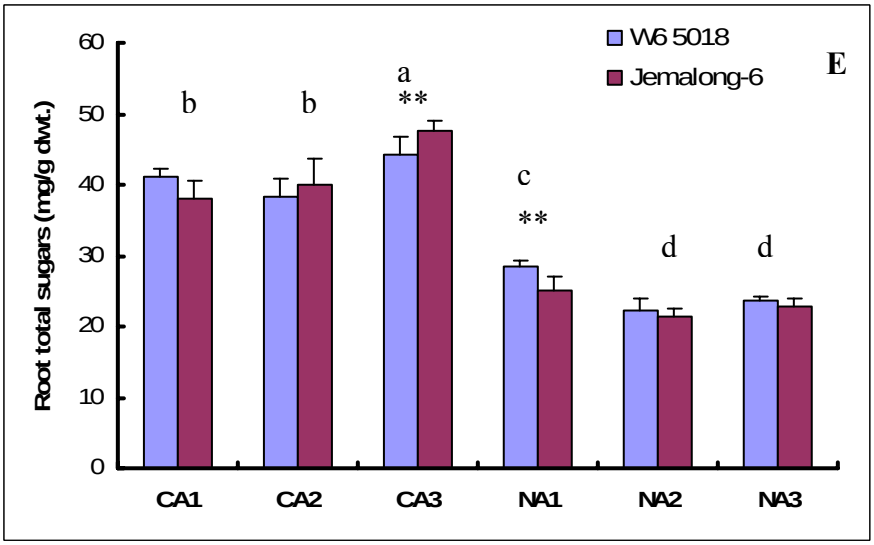


Figure 3. Concentrations of root glucose (A), fructose (B), sucrose (C), trehalose (D) and total sugars (E) in *M. truncatula* genotypes W6 5018 and Jemalong-6 at non-acclimation (NA1, NA2, NA3) and cold-acclimation (CA1, CA2, CA3) regimes. Vertical bars represent standard error of the mean (n=4). Growth regimes with the same letter are not significantly different according to Fisher's protected LSD (α =0.05). Asterisks indicate significant differences between the genotypes at P <0.01 (*) and <0.001 (**) within a growth regime.

References

Bachmann, M., P. Matile, and F. Keller. 1994. Metabolism of the Raffinose Family Oligosaccharides in Leaves of Ajuga reptans L.(Cold Acclimation, Translocation, and Sink to Source Transition: Discovery of Chain Elongation Enzyme), pp. 1335-1345 Plant Physiology, Vol. 105. Am Soc Plant Biol.

Burke, M.J., R.G. Bryant, and C.J. Weiser. 1974. Nuclear Magnetic Resonance of Water in Cold Acclimating Red Osier Dogwood Stem Plant Physiol. 54:392-398.

Castillo, E., B. De Lumen, P. Reyes, and H. De Lumen. 1990. Raffinose synthase and galactinol synthase in developing seeds and leaves of legumes. Journal of Agricultural and Food Chemistry 38:351-355.

Castonguay, Y., P. Nadeau, and R. Michaud. 1998. Differential accumulation of oligosaccharides and freezing tolerance of alfalfa. Plant Cold Hardiness. Plenum Press, New York:293–299.

Castonguay, Y., P. Nadeau, P. Lechasseur, and L. Chouinard. 1995. Differential Accumulation of Carbohydrates in Alfalfa Cultivars of Contrasting Winterhardiness. Crop Science 35:509.

Chen, H., and P. Li. 1980. Biochemical Changes in Tuber-bearing Solanum Species in Relation to Frost Hardiness during Cold Acclimation 1. Plant Physiology 66:414-421.

Cook, D. 1999. Medicago truncatula—a model in the making! Commentary. Current Opinion in Plant Biology 2:301-304.

Cunningham, S.M., P. Nadeau, Y. Castonguay, S. Laberge, and J.J. Volenec. 2003. Raffinose and Stachyose Accumulation, Galactinol Synthase Expression, and Winter Injury of Contrasting Alfalfa Germplasms. Crop Sci 43:562-570.

Dijken, A., H. Schluepmann, and S. Smeekens. 2004. Arabidopsis Trehalose-6-Phosphate Synthase 1 Is Essential for Normal Vegetative Growth and Transition to Flowering 1. Plant Physiology 135:969-977.

Dionne, J., Y., Castonguay, P. Nadeau, and Y. Desjardins. 2001. Freezing tolerance and carbohydrate changes during cold acclimation of green-type annual bluegrass(Poa annua L.) ecotypes. Crop science 41:443-451.

Elbein, A.D., Y.T. Pan, I. Pastuszak, and D. Carroll. 2003. New insights on trehalose: a multifunctional molecule. Glycobiology 13:17R-27.

Garg, A., J. Kim, T. Owens, A. Ranwala, Y. Choi, L. Kochian, and R. Wu. 2002. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proceedings of the National Academy of Sciences 99:15898-15903.

Gomez, L., S. Baud, A. Gilday, Y. Li, and I. Graham. 2006. Delayed embryo development in the *Arabidopsis* trehalose-6-phosphate synthase 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. The Plant Journal 46:69-84.

Graham, D., and B. Patterson. 1982. Responses of Plants to Low, Nonfreezing Temperatures: Proteins, Metabolism, and Acclimation. Annual Review of Plant Physiology 33:347-372.

Guy, C. 1990. Cold Accelimation and Freezing Stress Tolerance: Role of Protein Metabolism. Annual Reviews in Plant Physiology and Plant Molecular Biology 41:187-223.

Guy, C., J. Huber, and S. Huber. 1992. Sucrose Phosphate Synthase and Sucrose Accumulation at Low Temperature 1. Plant Physiology 100:502-508.

Hekneby, M., M. Antolín, and M. Sánchez-Díaz. 2001. Cold response of annual Mediterranean pasture legumes. Options Méditerranéennes. Série A: Séminaires Méditerranéens (CIHEAM).

Hekneby, M., M.C. Antolin, and M. Sanchez-Diaz. 2006. Frost resistance and biochemical changes during cold acclimation in different annual legumes. Environmental and Experimental Botany 55:305-314.

Hurry, V., A. Strand, M. Tobiaeson, P. Gardestrom, and G. Oquist. 1995. Cold Hardening of Spring and Winter Wheat and Rape Results in Differential Effects on Growth, Carbon Metabolism, and Carbohydrate Content, pp. 697-706 Plant Physiology, Vol. 109. Am Soc Plant Biol.

Hurry, V.M., G. Malmberg, P. Gardestrom, and G. Oquist. 1994. Effects of a Short-Term Shift to Low Temperature and of Long-Term Cold Hardening on Photosynthesis and Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase and Sucrose Phosphate Synthase Activity in Leaves of Winter Rye (Secale cereale L.). Plant Physiol. 106:983-990.

Jang, I.-C., S.-J. Oh, J.-S. Seo, W.-B. Choi, S.I. Song, C.H. Kim, Y.S. Kim, H.-S. Seo, Y.D. Choi, B.H. Nahm, and J.-K. Kim. 2003. Expression of a Bifunctional Fusion of the Escherichia coli Genes for Trehalose-6-Phosphate Synthase and Trehalose-

6-Phosphate Phosphatase in Transgenic Rice Plants Increases Trehalose Accumulation and Abiotic Stress Tolerance without Stunting Growth. Plant Physiol. 131:516-524.

Kacperska, A. 1993. Water potential alterations. A prerequisite or a triggering stimulus for the development of freezing tolerance in overwintering herbaceous plant?, pp. 73-91 Advances in Plant Cold Hardiness. CRC Press.

Kacperska, A., and L. Kulesza. 1987. Frost resistance of winter rape leaves as related to the changes in water potential and growth capability. Physiologia Plantarum 71:483-488.

Kacperska-Palacz, A. 1978. Mechanism of cold acclimation in herbaceous plants. Plant Cold Hardiness and Freezing Stress 1:139-152.

Klotke, J., J. Kopka, N. Gatzke, and A. Heyer. 2004. Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of Arabidopsis thaliana with contrasting cold adaptation-evidence for a role of raffinose in cold acclimation. Plant, Cell & Environment 27:1395-1404.

Lineberger, R.D., and P.L. Steponkus. 1980. Cryoprotection by Glucose, Sucrose, and Raffinose to Chloroplast Thylakoids Plant Physiol. 65:298-304.

Madore, M. 1995. Catabolism of raffinose family oligosaccharides by vegetative sink tissues. In M.A. Madore and W. J. Lucas(eds.) Carbon partitioning and source-sink Interactions in plants. American Society of plant Physiologists, p. 204-214.

Morsy, M., L. Jouve, J. Hausman, L. Hoffmann, and J. Stewart. 2007. Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (Oryza

sativa L.) genotypes contrasting in chilling tolerance. Journal of Plant Physiology 164:157-167.

Penna, S. 2003. Building stress tolerance through over-producing trehalose in transgenic plants. Trends in Plant Science 8:355-357.

Ramon, M., and F. Rolland. 2007. Plant development: introducing trehalose metabolism. Trends in Plant Science 12:185-188.

Sakai, A., and W. Larcher. 1987. Frost survival of plants. Responses and adaptation to freezing stress. Ecological Studies 62:1-346.

Sánchez-Díaz, M., M. Hekneby, and M. Antolín. 2000. Cold tolerance of forage legumes growing in controlled continental Mediterranean conditions. Cahiers Options Méditerranéennes 45:265-270.

Sangwan, V. B. L. Orvar, and R. S. Dhindsa. 2002. Early events during low temperature signaling.Paul H. Li and E. Tapio Palva. In Plant Cold Hardiness. pp 43-53.

Santarius, K. 1982. The mechanism of cryoprotection of biomembrane systems by carbohydrates. Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implications 2:475-486.

Sprenger, N., and F. Keller. 2000. Allocation of raffinose family oligosaccharides to transport and storage pools in Ajuga reptans: the roles of two distinct galactinol synthases. The Plant Journal 21:249-258.

Steponkus, P. 1984. Role of the Plasma Membrane in Freezing Injury and Cold Acclimation. Annual Reviews in Plant Physiology 35:543-584.

Stitt, M., and V. Hurry. 2002. A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in Arabidopsis. Current Opinion in Plant Biology 5:199-206.

Sturm, A. 1999. Invertases. Primary Structures, Functions, and Roles in Plant Development and Sucrose Partitioning, pp. 1-8 Plant Physiology, Vol. 121. Am Soc Plant Biol.

Thomashow, M. 1999. PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms. Annual Reviews in Plant Physiology and Plant Molecular Biology 50:571-599.

Thoquet, P., M. Gherardi, E. Journet, A. Kereszt, J. Ane, J. Prosperi, and T. Huguet. 2002. The molecular genetic linkage map of the model legume. Medicago truncatula:1–13.

Tronsmo, A. M., G.Kobro, S. Morgenlie, and R. Flengsrud. 1993. Carbohydrate content and glycosidase activities following cold hardening in two grass species. Physiologia Plantarum 88:689-695.

Uemura, M., G. Warren, and P. Steponkus. 2003. Freezing Sensitivity in the sfr4 Mutant of Arabidopsis Is Due to Low Sugar Content and Is Manifested by Loss of Osmotic Responsiveness. Plant Physiology 131:1800-1807.

Veluthambi, K., S. Mahadevan, and R. Maheshwari. 1982. Trehalose Toxicity in Cuscuta reflexa 1 Cell Wall Synthesis Is Inhibited upon Trehalose Feeding. Plant Physiology 70:686-688.

Wingler, A. 2002. The function of trehalose biosynthesis in plants. Phytochemistry 60:437-440.

Xin, Z., and J. Browse. 2000. Cold comfort farm: the acclimation of plants to freezing temperatures. Plant, Cell & Environment 23:893-902.

Yoshida, M., J. Abe, M. Moriyama, S. Shimokawa, and Y. Nakamura. 1997. Seasonal changes in the physical state of crown water associated with freezing tolerance in winter wheat. Physiologia Plantarum 99:363-370.

CHAPTER 5. GENERAL CONCLUSIONS

We characterize cold acclimation in *M. truncatula* as a biphasic process based on the two distinct levels of freezing tolerance observed with response to temperature treatments in this study. This is similar to what has been reported for winter hardy alfalfa. The two accessions used had different injury levels after exposure to freezing temperature treatments used in the study. Given the differential response of the two genotypes used in the study, it may valuable to screen other accessions to determine the overall variation in cold tolerance among *M. truncatula* accessions.

The two genotypes, Jemalong-6 and W6 5018, had increased freezing tolerance with response to cold-acclimation treatments. Simulated autumn conditions reduced stem length in *M. truncatula*, the response associated with fall dormancy in alfalfa. Stem length and number of leaves per stem were reduced in Jemalong-6 and W6 5018 with the start of different acclimation treatments during the first three weeks. These results indicate that differences in cold tolerances in the species may be partly relating to cultivars differences in their ability to acclimation in cold temperatures. Therefore, *M. truncatula* may possibly be used as a model for understanding genetics of cold tolerance in other legume crops.

Wide variation in root and shoot dry matter accumulation revealed the presence of exploitable yield variation among *M. truncatula* accessions. The majority of the accessions produced higher dry matter in roots and shoots under the simulated autumn condition, patterned after 20-years Ames's Iowa autumn temperature and photoperiod than in control. These results indicate that *M. truncatula* have considerable variation in cold tolerance abilities. Based on the growth assessment in autumn condition and genetic

difference for biomass yield, some of the accessions have potential to be grown as a short-season winter forage crop for fall harvest upon evaluation in the field conditions. In general, it appears that stem length can be used as an indicator for selection of high yielding accessions, based on positive association of the two traits observed under control and modulated fall conditions. Time to first flower, the only trait associated consistently with differences among geographical locations, was delayed by simulated autumn condition showing a plastic response with temperature treatments and proved a clear evidence for adaptation ability of the species to diverse locations.

Soluble sugars and total soluble sugars concentrations varied considerably between cold-acclimation and nonacclimation regimes and also among the CA regimes which may reveal that these physiological traits are indicative of cold tolerance in the species. However, further research is required to establish their values as a screening procedure for cold acclimation in *M. truncatula*. The concentration of sugars improved the osmotic regulation of leaf tissues and membrane stability as indicated by their positive correlation with the percent water content in the leaf and freezing tolerance. Among all the soluble sugars, raffinose was found in cold-acclimated tissues while it remained undetected in non-acclimated tissues and seems to be the best indicator for cold acclimation in *M. truncatula.* However, we did not see cultivar difference in leaf raffinose concentration although it was observed in cold acclimated stem, higher concentrations after acclimated at lower temperature. Further studies focused on evaluation of differences in raffinose concentrations in a set of genotypes with varying cold tolerance after they were exposed to subzero temperature for a longer duration. Correlation between the raffinose concentration and cold tolerance of genotypes may

elucidate if raffinose can be used as a screening tool for freezing tolerance in *M. truncatula*.

ACKNOWLEDGEMENTS

First and foremost, I am grateful to Dr. Allen D. Knapp, my major professor for taking me on as a graduate student in the Crop Production and Physiology Program at Iowa State University. His consistent guidance and insightful thoughts were invaluable to me to be able to explain my research thoroughly and thoughtfully. I would like to thank my second major professor, Dr. Charles E. Brummer for his knowledge and ideas for the success of this work and for his endless effort in helping me improve my work. My appreciations are due to Dr. Rajeev Arora for providing guidance and support and allowing me to use his laboratory for a part of this study. Many thanks to the Agronomy faculty members, Dr. Kenneth J. Moore and Dr. Jeremy Singer, who served as my POS committee and for being available every time I approached them with questions and for their invaluable time reviewing this work for its improvement.

Special thanks to my husband for his tremendous love, support and patience through all these years. Thanks to my mother, Mrs. Bimala Singh K.C. and my sisters for their encouragement and unconditional love. Thanks to my extended family in the US and in Nepal for their well wishes.

I am especially thankful to Maria Hartt, Crop Production and Physiology laboratory Manager, who was instrumental in all aspects of this research. Thanks to friends of the Department of Agronomy and the Nepalese Student Association (ISU), for their friendship, help and for all the wonderful times we shared together.